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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		Attorney's Docket Number 05394.0011 U.S. Application No. 09/673476
International Application. No. PCT/IB99/00740	International Filing Date April 16, 1999	Priority Date Claimed April 16, 1998

Title of Invention:
A METHOD FOR ISOLATING A POLYNUCLEOTIDE OF INTEREST FROM THE GENOME OF A MYCOBACTERIUM USING A BAC-BASED DNA LIBRARY. APPLICATION TO THE DETECTION OF MYCOBACTERIA

Applicant(s) For DO/EO/US:
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [X] Annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:
    a. [ ] Verified Small Entity Statement.
    b. [ ] Copy of Notification of Missing Requirements.
    c. [X] Copy of Cover Page of WIPO Publication (1 sheet).
    d. [X] Sequence Listing (11 pages).

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17. [X] The following fees are submitted:

	CALCULATIONS
Basic National Fee (37 CFR 1.492(a)(1)-(5)):	
Search Report has been prepared by the EPO or JPO.........$860.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482)....................................$690.00	
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))......................$760.00	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....................$1,000.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..............................$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$ 860.00**
Surcharge of $130.00 for furnishing the oath or declaration later than **[ ] 20 [ ] 30** months from the earliest claimed priority date (37 CFR 1.492(e)).	$

Claims	Number Filed	Number Extra	Rate	
Total Claims	55 -20=	35	X $18.00	$ 630.00
Independent Claims	4 - 3=	1	X $80.00	$ 80.00
Multiple dependent claim(s) (if applicable)			+$270.00	$ 270.00
TOTAL OF ABOVE CALCULATIONS =				**$1,840.00**
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)				$
SUBTOTAL =				**$1,840.00**
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$
TOTAL NATIONAL FEE =				**$1,840.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$
TOTAL FEES ENCLOSED =				**$1,840.00**
Amount to be refunded				$
charged				$

a. [X] A check in the amount of **$1,840.00** to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _________ in the amount of $______________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-0916. A duplicate copy of this sheet is enclosed.

The Commissioner is hereby authorized to charge any other fees due under 37 C.F.R. §1.16 or §1.17 during the pendency of this application to our Deposit Account No. 06-0916.

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A METHOD FOR ISOLATING A POLYNUCLEOTIDE OF INTEREST FROM THE GENOME OF A MYCOBACTERIUM USING A BAC-BASED DNA LIBRARY~~. APPLICATION TO THE DETECTION~~ OF MYCOBACTERIA.

I. Background of the invention

The present invention pertains to a method for isolating a polynucleotide of interest that is present in the genome of a mycobacterium strain and/or is expressed by said mycobacterium strain and that is absent or altered in the genome of a different mycobacterium strain and/or is not expressed in said different mycobacterium strain, said method comprising the use of at least one clone belonging to a genomic DNA library of a given mycobaterium strain, said DNA library being cloned in a bacterial artificial chromosome (BAC). The invention concerns also polynucleotides identified by the above method, as well as detection methods for mycobacteria, particularly *Mycobacterium tuberculosis*, and kits using said polynucleotides as primers or probes. Finally, the invention deals with BAC-based mycobacterium DNA libraries used in the method according to the invention and particularly BAC-based *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG DNA libraries.

Radical measures are required to prevent the grim predictions of the World Health Organisation for the evolution of the global tuberculosis epidemic in the next century becoming a tragic reality. The powerful combination of genomics and bioinformatics is providing a wealth of information about the etiologic agent, *Mycobacterium tuberculosis*, that will facilitate the conception and development of new therapies. The start point for genome sequencing was the integrated map of the 4.4 Mb circular chromosome of the widely-used, virulent reference strain, *M. tuberculosis* H37Rv and appropriate cosmids were subjected to systematic shotgun sequence analysis at the Sanger Centre.

Cosmid clones (Balasubramanian et al., 1996; Pavelka et al., 1996) have played a crucial role in the *M. tuberculosis* H37Rv genome sequencing project. However, problems such as under-representation of certain regions of the chromosome, unstable inserts and the relatively small insert size complicated the production of a comprehensive set of canonical cosmids representing the entire genome.

II. Summary of the invention

In order to avoid the numerous technical constraints encountered in the state of the art, as decribed hereabove, when using genomic mycobacterial DNA libraries constructed in cosmid clones, the inventors have attempted to realize genomic mycobacterial DNA libraries in an alternative type of vectors, namely Bacterial Artificial Chromosome (BAC) vectors.

The success of this approach depended on whether the resulting BAC clones could maintain large mycobacterial DNA inserts. There are various reports describing the successful construction of a BAC library for eucaryotic organisms (Cai et al., 1995; Kim et al., 1996; Misumi et al., 1997; Woo et al., 1994; Zimmer et al., 1997) where inserts up to 725 kb (Zimmer et al., 1997) were cloned and stably maintained in the *E. coli* host strain.

Here, it is shown that, surprisingly, the BAC system can also be used for mycobacterial DNA, as 70% of the clones contained inserts in the size of 25 to 104 kb.

This is the first time that bacterial, and specifically mycobacterial, DNA is cloned in such BAC vectors.

In an attempt to obtain complete coverage of the genome with a minimal overlapping set of clones, a Bacterial Artificial Chromosome (BAC) library of *M. tuberculosis* was constructed, using the vector pBeloBAC11 (Kim et al., 1996) which combines a simple phenotypic screen for recombinant clones with the stable propagation of large inserts (Shizuya et al., 1992). The BAC cloning system is based on the *E. coli* F-factor, whose replication is strictly controlled and thus ensures stable maintenance of large constructs (Willets et al., 1987). BACs have been widely used for cloning of DNA from various eucaryotic species (Cai et al., 1995; Kim et al., 1996; Misumi et al., 1997; Woo et al., 1994; Zimmer et al., 1997). In contrast, to our knowledge this report describes the first attempt to use the BAC system for cloning bacterial DNA.

A central advantage of the BAC cloning system over cosmid vectors used in prior art is that the F-plasmid is present in only one or a maximum of two copies per cell, reducing the potential for recombination between DNA fragments and, more importantly, avoiding the lethal overexpression of cloned bacterial genes. However, the presence of the BAC as just a single copy means that plasmid DNA has to be extracted from a large volume of culture to obtain

sufficient DNA for sequencing and it is described here in the examples a simplified protocol to achieve this.

Further, the stability and fidelity of maintenance of the clones in the BAC library represent ideal characteristics for the identification of genomic differences possibly responsible for phenotypic variations in different mycobacterial species.

As it will be shown herein, BACs can be allied with conventional hybridization techniques for refined analyses of genomes and transcriptional activity from different mycobacterial species.

Having established a reliable procedure to screen for genomic polymorphisms, it is now possible to conduct these comparisons on a more systematic basis than in prior art using representative BACs throughout the chromosome and genomic DNA from a variety of mycobacterial species.

As another approach to display genomic polymorphisms, the inventors have also started to use selected H37Rv BACs for "molecular combing" experiments in combination with fluorescent *in situ* hybridization (Bensimon et al., 1994; Michalet et al., 1997). With such techniques the one skilled in the art is enabled to explore the genome of mycobacteria in general and of *M. tuberculosis* in particular for further polymorphic regions.

The availability of BAC-based genomic mycobacterial DNA libraries constructed by the inventors have allowed them to design methods and means both useful to identify genomic regions of interest of pathogenic mycobacteria, such as *Mycobacterium tuberculosis*, that have no counterpart in the corresponding non-pathogenic strains, such as *Mycobacterium bovis* BCG, and useful to detect the presence of polynucleotides belonging to a specific mycobacterium strain in a biological sample.

By a biological sample according to the present invention, it is notably intended a biological fluid, such as plasma, blood, urine or saliva, or a tissue, such as a biopsy.

Thus, a first object of the invention consists of a method for isolating a polynucleotide of interest that is present in the genome of a mycobacterium strain and/or is expressed by said mycobacterium strain and that is absent or altered in the genome of a different mycobacterium strain and/or is not expressed in said different mycobacterium strain, said method comprising the use of at least one clone belonging to a genomic DNA library of a given mycobaterium strain, said DNA library being cloned in a bacterial artificial chromosome (BAC).

The invention is also directed to a polynucleotide of interest that has been isolated according to the above method and in partoular a polynucleotide containing one or several Open Reading Frames (ORFs), for example ORFs encoding either a polypeptide involved in the pathogenicity of a mycobacterium strain or ORFs encoding Polymorphic Glycine Rich Sequences (PGRS).

Such polynucleotides of interest may serve as probes or primers in order to detect the presence of a specific myobacterium strain in a biological sample or to detect the expression of specific genes in a particular mycobacterial strain of interest.

The BAC-based genomic mycobacterial DNA libraries generated by the present inventors are also part of the invention, as well as each of the recombinant BAC clones and the DNA insert contained in each of said recombinant BAC clones.

The invention also pertains to methods and kits for detecting a specific mycobacterium in a biological sample using either at least one recombinant BAC clone or at least one polynucleotide according to the invention, as well as to methods and kits to detect the expression of one or several specific genes of a given mycobacterial strain present in a biological sample.

III. Brief description of the Figures.

In order to better understand the present invention, reference will be made to the appended figures which depicted specific embodiments to which the present invention is in no case limited in scope with.

Figures 1A and 1B : PCR-screening for unique BAC clones with specific primers for 2 selected genomic regions of the H37Rv chromosome, using 21 pools representating 2016 BACs (Figure 1A) and sets of 20 subpools from selected positive pools (Figure 1B).

Figure 2 : Pulsed-field gel electrophoresis gel of *Dra*I- cleaved BAC clones used for estimating the insert sizes of BACs.

Figure 3 : Minimal overlapping BAC map of *M. tuberculosis* H37Rv superimposed on the integrated physical and genetic map established by Philipp et al. (18). Y- and I- numbers show pYUB328 (2) and pYUB412 (16) cosmids which were shotgun sequenced during the H37Rv genome sequencing project. Y-cosmids marked with * were shown in the integrated physical and genetic map

(18). Rv numbers show the position of representative BAC clones relative to sequenced Y- and I- clones. Squared Rv numbers show BACs which were shotgun sequenced at the Sanger Centre.

Figures 4A and 4B : Ethidium bromide stained gel (Figure 4A) and corresponding Southern blot (Figure 4B) of *Eco*RI and *Pvu*II digested Rv58 DNA hybridized with ^{32}P labeled genomic DNA preparations from *M. tuberculosis* H37Rv, *M. bovis* ATCC 19210 and *M. bovis BCG* Pasteur.

Figure 5 : Organisation of the ORFs in the 12.7 kb genomic region present in *M. tuberculosis* H37Rv but not present in *M. bovis* ATCC 19210 and *M. bovis* BCG Pasteur. Arrows show the direction of transcription of the putative genes. Positions of *Eco*RI and *Pvu*II restriction sites are shown. Vertical dashes represent stop codons. The 11 ORFs correspond to the ORFs MTCY277.28 to MTCY277.38 / accession number Z79701 -EMBL Nucleotide Sequence Data Library. The junction sequences flanking the polymorphic region are shown.

Figure 6 : Variation in the C-terminal part of a PE-PGRS open reading frame in *M. tuberculosis* strain H37Rv relative to *M. bovis* BCG strain Pasteur.

The numbers on the right side of the Figure denote the position of the end nucleotides, taking as the reference the *M. tuberculosis* genome.

Figure 7 : Polynucleotide sequence next to the HindIII cloning site in the BAC vector pBeloBAC11 (Kim et al., 1996) used to clone the inserts of the BAC-based myobacterial genomic DNA library according to the invention.

NotI : location of the NotI restriction sites.

Primer T7-BAC1 : nucleotide region recognized by the T7-BAC1 primer shown in Table 1.

T7 promoter : location of the T7 promoter region on the pBeloBac11 vector.

Primer T7-Belo2 : nucleotide region recognized by the T7-Belo2 pimer shown in Table 1.

Hind III : the HindIII cloning site used to clone the genomic inserts in the pBeloBAC11 vector.

SP6-Mid primer : nucleotide region recognized by the SP6 Mid primer shown in Table 1.

SP6-BAC1 primer : nucleotide region recognized by the SP6 BAC1 primer shown in Table 1.

SP6 promoter : location of the SP6 promoter region onthe pBeloBac11 vector.

IV. Detailed description of the preferred embodiments.

As already mentioned hereinbefore, the present invention is directed to a method for isolating a polynucleotide of interest that is present in the genome of a mycobacterium strain and/or is expressed by said mycobacterium strain and that is absent or altered in the genome of a different mycobacterium strain and/or is not expressed in said different mycobacterium strain, said method comprising the use of at least one clone belonging to a genomic DNA library of a given mycobaterium strain, said DNA library being cloned in a bacterial artificial chromosome (BAC) type vector.

For this purpose, the inventors have constructed several BAC-based mycobacterial genomic DNA libraries that may be used in order to perform the above described method.

Because it is the first time that mycobacterial genomic DNA has been successfully cloned in BAC type vectors, and because these DNA libraries are then novel and nonobvious, an object of the present invention consists in a myobacterial genomic DNA library cloned in such a BAC type vector.

As an illustrative example, a BAC-based DNA library of *Mycobacterium tuberculosis* has been realized. Forty-seven cosmids chosen from the integrated map of the 4.4 Mb circular chromosome (Philipp et al., 1996a) were shotgun-sequenced during the initial phase of the H37Rv genome sequence project. The sequences of these clones were used as landmarks in the construction of a minimally overlapping BAC map. Comparison of the sequence data from the termini of 420 BAC clones allowed us to establish a minimal overlapping BAC map and to fill in the existing gaps between the sequence of cosmids. As well as using the BAC library for genomic mapping and sequencing, we also tested the system in comparative genomic experiments in order to uncover differences between two closely related mycobacterial species. As shown in a previous study (Philipp et al., 1996b), *M. tuberculosis*, *M. bovis* and *M. bovis* BCG, specifically BCG Pasteur strain, exhibit a high level of global genomic conservation, but certain polymorphic regions were also detected. Therefore, it was of great interest to find a reliable, easy and rapid way to exactly localize polymorphic regions in mycobacterial genomes using selected BAC clones. This approach was validated by determining the exact size and location of the polymorphisms in the genomic region of *Dra*I fragment Z4 (Philipp et al., 1996b), taking advantage of the availability of an appropriate BAC clone covering the polymorphic region and

the H37Rv genome sequence data. This region is located approximately 1.7 Mb from the origin of replication.

The Bacterial Artificial Chromosome (BAC) cloning system is capable of stably propagating large, complex DNA inserts in *Escherichia coli*. As part of the *Mycobacterium tuberculosis* H37Rv genome sequencing project, a BAC library was constructed in the pBeloBAC11 vector and used for genome mapping, confirmation of sequence assembly, and sequencing. The library contains about 5000 BAC clones, with inserts ranging in size from 25 to 104 kb, representing theoretically a 70 fold coverage of the *M. tuberculosis* genome (4.4 Mb). A total of 840 sequences from the T7 and SP6 termini of 420 BACs were determined and compared to those of a partial genomic database. These sequences showed excellent correlation between the estimated sizes and positions of the BAC clones and the sizes and positions of previously sequenced cosmids and the resulting contigs. Many BAC clones represent linking clones between sequenced cosmids, allowing full coverage of the H37Rv chromosome, and they are now being shotgun-sequenced in the framework of the H37Rv sequencing project. Also, no chimeric, deleted or rearranged BAC clones were detected, which was of major importance for the correct mapping and assembly of the H37Rv sequence. The minimal overlapping set contains 68 unique BAC clones and spans the whole H37Rv chromosome with the exception of a single gap of ~ 150 kb. As a post-genomic application, the canonical BAC set was used in a comparative study to reveal chromosomal polymorphisms between *M. tuberculosis*, *M. bovis* and *M. bovis* BCG Pasteur, and a novel 12.7 kb segment present in *M. tuberculosis* but absent from *M. bovis* and *M. bovis* BCG was characterized. This region contains a set of genes whose products show low similarity to proteins involved in polysaccharide biosynthesis. The H37Rv BAC library therefore provides the one skilled in the art with a powerful tool both for the generation and confirmation of sequence data as well as for comparative genomics and a plurality of post-genomic applications.

The above described BAC-based *Mycobacterium tuberculosis* genomic DNA library is part of the present invention and has been deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) on November 19, 1997 under the accession number I-1945.

Another BAC-based DNA library has been constructed with the genomic DNA of *Mycobacterium bovis* BCG, Pasteur strain, and said DNA library has

been deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) on June 30, 1998 under the accession number I-2049.

Thus, as a specific embodiment of the above described method for isolating a polynucleotide of interest said method makes use of at least one BAC-based DNA library that has beeen constructed from the genomic DNA of *Mycobacterium tuberculosis*, more specifically of the H37Rv strain and particularly of the DNA library deposited in the accession number I-1945.

In another specific embodiment of the above described method for isolating a polynucleotide of interest said method makes use of at least one BAC-based DNA library has beeen constructed from the genomic DNA of *Mycobacterium bovis* BCG, more specifically of the Pasteur strain and particularly of the DNA library deposited in the accession number I-2049.

In more details, the method according to the invention for isolating a polynucleotide of interest may comprise the following steps :

a) isolating at least one polynucleotide contained in a clone of a BAC-based DNA library of mycobacterial origin;

b) isolating :

- at least one genomic or cDNA polynucleotide from a mycobacterium, said mycobacterium belonging to a strain different from the strain used to construct the BAC-based DNA library of step a); or alternatively

- at least one polynucleotide contained in a clone of a BAC-based DNA library prepared from the genome of a mycobacterium that is different from the mycobacterium used to construct the BAC-based DNA library of step a);

c) hybridizing the at least one polynucleotide of step a) to the at least one polynucleotide of step b);

d) selecting the at least one polynucleotide of step a) that has not formed a hybrid complex with the at least one polynucleotide of step b);

e) characterizing the selected polynucleotide.

Following the above procedure, the at least one polynucleotide of step a) may be prepared as follows :

1) digesting at least one recombinant BAC clone by an appropriate resctriction endonuclease in order to isolate the polynucleotide insert of interest from the vector genetic material;

2) optionally amplifying the resulting polynucleotide insert;

3) optionally digesting the polynucleotide insert of step 1) or step 2) with at least one restriction endonuclease.

The above method of the invention allows the one skilled in the art to perform comparative genomics between different strains or species of mycobacteria cells, for example between pathogenic strains or species and their non pathogenic strains or species counterparts, as it is the illustrative case for the genomic comparison between *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG that is described herein in the examples.

Restriction digests of a given clone of a BAC library according to the invention may be blotted to membranes, and then probed with radiolabeled DNA form another strain or another species of mycobacteria, allowing the one skilled in the art to identify, characterize and isolate a polynucleotide of interest that may be involved in important metabolical and/or physiological pathways of the mycobacterium under testing, such as a polynucleotide functionally involved in the pathogenicity of said given mycobacteria for its host organism.

More specifically, the inventors have shown in Example 6 that when restriction digests of a given clone of the BAC library identified by the CNCM accession number I-1945 are blotted to membranes and then probed with radiolabeled total genomic DNA from, for example, *Mycobacterium bovis* BCG Pasteur, it is observed that restriction fragments that fail to hybridize with the *M. bovis* BCG Pasteur DNA are absent from its genome, hence identifying polymorphic regions between *M. bovis* BCG Pasteur and *M. tuberculosis* H37Rv.

Thus, a further object of the present invention consists in a polynucleotide of interest that has been isolated according to the method described herein before.

In Example 6, a polynucleotide of approximately 12.7 kilobases has been isolated that is present in the genome of *M. tuberculosis* but is absent of the genome of *M. bovis* BCG. This polynucleotide of interest contains 11 ORFs that may be involved in polysaccharide biosynthesis. In particular, two of said ORFs are of particular interest, namely ORF6 (MTCY277.33; Rv1511) that encodes a protein that shares significant homology with bacterial GDP-D-mannose dehydratases, whereas the protein encoded by ORF7 (MTCY277.34; Rv1512) shares significant homology with a nucleotide sugar epimerase. As polysaccharide is a major constituent of the mycobacterial cell wall, these deleted genes may cause the cell wall of *M. bovis* BCG to differ from that of *M. tuberculosis*, a fact that may have important consequences for both the immune

response to *M. bovis* BCG and virulence. Detection of such a polysaccharide is of diagnostic interest and possibly useful in the design of tuberculosis vaccines.

Consequently, the polynucleotide of interest obtained following the method according to the invention may contain at least one ORF, said ORF preferably encoding all or part of a polypeptide involved in an important metabolical and/or physiological pathway of the mycobacteria under testing, and more specifically all or part of a polypeptide that is involved in the pathogenicity of the mycobacteria under testing, such as for example *Mycobacterium tuberculosis*, and more generally mycobacteria belonging to the *Mycobacterium tuberculosis* complex.

The *Mycobacterium tuberculosis* complex has its usual meaning, i.e. the complex of mycobacteria causing tuberculosis which are *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti* and the vaccine strain *Mycobacterium bovis* BCG.

An illustrative polynucleotide of interest according to the present invention comprises all or part of the polynucleotide of approximately 12.7 kilobases that is present in the genome of *M. tuberculosis* but is absent from the genome of *M. bovis* BCG disclosed hereinbefore. This polynucleotide is contained in clone Rv58 of the BAC DNA library I-1945.

Generally, the invention also pertains to a purified polynucleotide comprising the DNA insert contained in a recombinant BAC vector belonging to a BAC-based mycobacterial genomic DNA library, such as for example the I-1945 BAC DNA library.

Advantageously, such a polynucleotide has been identified according to the method of the invention.

Such a polynucleotide of interest may be used as a probe or a primer useful for specifically detecting a given mycobacterium of interest, such as *Mycobacterium tuberculosis* or *Mycobacterium bovis* BCG.

More specifically, the invention then deals with a purified polynucleotide useful as probe or a primer comprising all or part of the nucleotide sequence SEQ ID N°1.

The location, on the *Mycobacterium tuberculosis* chromosome, of the above polynucleotide of sequence SEQ ID N°1 has now been ascribed to begin, at its 5'end at nucleotide at position nt 1696015 and to end, at its 3'end, at nucleotide at position nt 1708746.

For diagnostic purposes, this 12.7 kb deletion should allow a rapid PCR screening of tubercle isolates to identify whether they are bovine or human strains. The primers listed in Table 1 are flanking the deleted region and give a 722 bp amplicon in *M. bovis* or *M. bovis* BCG strains, but a fragment of 13,453 bp in *M. tuberculosis* that is practically impossible to amplify under the same PCR conditions. More importantly, assuming that some of the gene products from this region represent proteins with antigenic properties, it could be possible to develop a test that can reliably distinguish between the immune response induced by vaccination with *M. bovis* BCG vaccine strains and infection with *M. tuberculosis* or that the products (e.g. polysaccharides) are specific immunogens.

The invention also provides for a purified polynucleotide useful as a probe or as a primer, said polynucleotide being chosen in the following group of polynucleotides :

a) a polynucleotide comprising at least 8 consecutive nucleotides of the sequence SEQ ID N°1;

b) a polynucleotide whose sequence is fully complementary to the sequence of the polynucleotide defined in a);

c) a polynucleotide that hybridizes under stringent hybridization conditions with the polynucleotide defined in a) or with the polynucleotide defined in b).

For the purpose of defining a polynucleotide or oligonucleotide hybridizing under stringent hybridization conditions, such as above, it is intended a polynucleotide that hybridizes with a reference polynucleotide under the following hybridization conditions.

The hybridization step is realized at 65°C in the presence of 6 x SSC buffer, 5 x Denhardt's solution, 0,5% SDS and 100µg/ml of salmon sperm DNA.

For technical information, 1 x SSC corresponds to 0.15 M NaCl and 0.05M sodium citrate; 1 x Denhardt's solution corresponds to 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin.

The hybridization step is followed by four washing steps :

- two washings during 5 min, preferably at 65°C in a 2 x SSC and 0.1%SDS buffer,
- one washing during 30 min, preferably at 65°C in a 2 x SSC and 0.1% SDS buffer,
- one washing during 10 min, preferably at 65°C in a 0.1 x SSC and 0.1%SDS buffer.

A first illustrative useful polynucleotide that is included in the polynucleotide of sequence SEQ ID N°1 is the polynucleotide of sequence SEQ ID N°2 that corresponds to the Sp6 end-sequence of SEQ ID N°1.

A second illustrative useful polynucleotide that is included in the polynucleotide of sequence SEQ ID N°1 is the polynucleotide of sequence SEQ ID N°3 that corresponds to the T7 end-sequence of SEQ ID N°1, located on the opposite strand.

The polynucleotide of sequence SEQ ID N°1 contains 11 ORFs, the respective locations of which, taking into account the orientation of each ORF on the chromosome, on the sequence of the *Mycobacterium tunerculosis* chromosome, is given hereafter :

- The location of ORF1 is comprised between nucleotide at position nt 1695944 and nucleotide at position nt1696441.
- The location of ORF2 is comprised between nucleotide at position nt 1696728 and nucleotide at position nt1697420.
- The location of ORF3 is comprised between nucleotide at position nt 1698096 and nucleotide at position nt1699892. ORF3 probably encodes a protein having the characteristics of a membrane protein.
- The location of ORF4 is comprised between nucleotide at position nt 1700210 and nucleotide at position nt1701088.
- The location of ORF5 is comprised between nucleotide at position nt 1701293 and nucleotide at position nt1702588. ORF5 encodes a protein having the characteristics of a membrane protein.
- The location of ORF6 is comprised between nucleotide at position nt 1703072 and nucleotide at position nt1704091. ORF6 encodes a protein having the characteristics of a GDP-D-mannose dehydratase.
- The location of ORF7 is comprised between nucleotide at position nt 1704091 and nucleotide at position nt1705056. ORF7 encodes a protein having the characteristics of a nucleotide sugar epimerase involved in colanic acid biosynthesis.
- The location of ORF8 is comprised between nucleotide at position nt 1705056 and nucleotide at position nt1705784.
- The location of ORF9 is comprised between nucleotide at position nt 1705808 and nucleotide at position nt1706593. ORF9 encodes a protein having the characteristics of colanic acid biosynthesis glycosyl transferase.

- The location of ORF10 is comprised between nucleotide at position nt 1706631 and nucleotide at position nt1707524.
- The location of ORF11 is comprised between nucleotide at position nt 1707530 and nucleotide at position nt1708648. ORF11 encodes a protein similar to a spore coat polysaccharide biosynthesis.

A polynucleotide of interest obtained by the above-disclosed method according to the invention may also contain at least one ORF that encodes all or part of acidic, glycine-rich proteins, belonging to the PE and PPE families, whose genes are often clustered and based on multiple copies of the polymorphic repetitive sequences. The names PE and PPE derive from the fact that the motifs ProGlu (PE, positions 8, 9) and ProProGlu (PPE, positions 7 to 9) are found near the N-terminus in almost all cases. The PE protein family all have a highly conserved N-terminal domain of ~110 amino acid residues, that is predicted to have a globular structure, followed by a C-terminal segment which varies in size, sequence and repeat copy number. Phylogenetic analysis separated the PE family into several groups, the larger of which is the highly repetitive PGRS class containing 55 members whereas the other groups share very limited sequence similarity in their C-terminal domains. The predicted molecular weights of the PE proteins vary considerably as a few members only contain the ~110 amino acid N-terminal domain while the majority have C-terminal extensions ranging in size from 100 up to >1400 residues. A striking feature of the PGRS proteins is their exceptional glycine content (up to 50%) due to the presence of multiple tandem repetitions of GlyGlyAla or GlyGlyAsn motifs or variations thereof.

Like the PE family, the PPE protein family also has a conserved N-terminal domain that comprises ~180 amino acid residues followed by C-terminal segments that vary considerably in sequence and length. These proteins fall into at least three groups, one of which constitutes the MPTR class characterised by the presence of multiple, tandem copies of the motif AsnXGlyXGlyAsnXGly. The second subgroup contains a characteristic, well-conserved motif around position 350 (GlyXXSerValProXXTrp), whereas the other group contains proteins that are unrelated except for the presence of the common 180-residue PPE domain. C-terminal extensions may range in size from 00 up to 3500 residues.

One member of the PGRS sub-family, the WHO antigen 22T (Abou-Zeid et al., 1991), a 55kD protein capable of binding fibronectin, is produced during

disease and elicits a variable antibody response suggesting either that individuals mount different immune responses or that this PGRS-protein may not be produced in this form by all strains of *M. tuberculosis*. In other words, at least some PE_PGRS coding sequences encode for proteins that are involved in the recognition of *M. tuberculosis* by the immune system of the infected host. Therefore, differences in the PGRS sequences could represent the principal source of antigenic variation in the otherwise genetically and antigenically homogeneous bacterium.

By performing the method of the invention using the *M. tuberculosis* BAC based DNA library I-1945, the inventors have discovered the occurence of sequence differences between a given PGRS encoding ORF (ORF reference on the genomic sequence of *M. tuberculosis* Rv0746) of *M. tuberculosis* and its counterpart sequence in the genome of *M. bovis* BCG.

More precisely, the inventors have determined that one ORF contained in BAC vector N° Rv418 of the *M. tuberculosis* BCG I-1945 DNA library carries both base additions and base deletions when compared with the corresponding ORF in the genome of *M. bovis* BCG that is contained in the BAC vector N° X0175 of the *M. bovis* BCG I-2049 DNA libary. The variations observed in the base sequences correspond to variations in the C-terminal part of the aminoacid sequence of the PGRS ORF translation product.

As shown in Figure 6, an amino acid stretch of 9 residues in length is present in this *M. tuberculosis* PGRS (ORf reference Rv0746) and is absent from the ORF counterpart of *M. bovis* BCG, namely the following amino acid sequence:

NH_2-GGAGGAGGSSAGGGGAGGAGGAGGWLLGD-COOH.

Furthermore, Figure 6 shows also that an amino acid stretch of 45 residues in length is absent from this *M. tuberculosis* PGRS and is present in the ORF counterpart of *M. bovis* BCG, namely following amino acid sequence:

NH_2-GAGGIGGIGGNANGGAGGNGGTGGQLWGSGGAGVEGGAAL SVGDT-COOH.

Similar observations were made with PPE ORF Rv0442, which showed a 5 codon deletion relative to a *M. bovis* amino acid sequence.

Given that the polymorphism associated with the PE-PGRS or PEE ORFS resulted in extensive antigenic variability or reduced antigen presentation, this would be of immense significance for vaccine design, for understanding

protective immunity in tuberculosis and, possibly, explain the varied responses seen in different BCG vaccination programmes.

There are several striking parallels between the PGRS proteins and the Epstein-Barr virus-encoded nuclear antigens (EBNA). Both polypeptide families are glycine-rich, contain Gly-Ala repeats that represent more than one third of the molecule, and display variation in the length of the repeat region between different isolates. The Gly-Ala repeat region of EBNA1 has been shown to function as a *cis*-acting inhibitor of antigen processing and MHC class I-restricted antigen presentation (Levitskaya et al., 1995). The fact that MHC class I knock-out mice are extremely suscepible to *M. tuberculosis* underlines the importance of MHC class I antigen presentation in protection against tuberculosis. Therefore, it is possible that the PE/PPE protein family also play some role in inhibiting antigen presentation, allowing the bacillus to hide from the host's immune system.

As such the novel and nonobvious PGRS polynucleotide from *M. bovis* which is homolog to the *M. tuberculosis* ORF Rv0746, and which is contained in the BAC clone N° X0175 (See Table 4 for SP6 and T7 end-sequences of clone n° X0175) of the I-2049 *M. bovis* BCG BAC DNA library is part of the present invention, as it represents a starting material in order to define specific probes or primers useful for detection of antigenic variability in mycobacterial strains, possible inhibition of antigen processing as well as to differentiate *M. tuberculosis* from *M. bovis* BCG.

Thus, a further object of the invention consists in a polynucleotide comprising the sequence SEQ ID N°4.

Polynucleotides of interest have been defined by the inventors as useful detection tools in order to differentiate *M. tuberculosis* from *M. bovis* BCG. Such polynucleotides are contained in the 45 aminoacid length coding sequence that is present in *M. bovis BCG* but absent from *M. tuberculosis*. This polynucleotide has a sequence beginning (5'end) at the nucleotide at position nt 729 of the sequence SEQ ID N°4 and ending (3'end) at the nucleotide in position nt 863 of the sequence SEQ ID N°4.

Thus, part of the present invention is also a polynucleotide which is chosen among the following group of polynucleotides :

a) a polynucleotide comprising at least 8 consecutive nucleotides of the nucleotide sequence SEQ ID N°5 ;

b) a polynucleotide which sequence is fully complementary to the sequence of the polynucleotide defined in a) ;

c) a polynucleotide that hybridizes under stringent hybridization conditions with the polynucleotide defined in a) or with the polynucleotide defined in b).

The stringent hybridization conditions for the purpose of defining the above disclosed polynucleotide are defined herein before in the specification.

The invention also provides for a BAC-based *Mycobacterium tuberculosis* strain H37Rv genomic DNA library that has been deposited in the Collection Nationale de Cultures de Microorganismes on November 19, 1997 under the accession number I-1945.

A further object of the invention consists in a recombinant BAC vector which is chosen among the group consisting of the recombinant BAC vectors belonging to the BAC-based DNA library I-1945.

Generally, a recombinant BAC vector of interest may be chosen among the following set or group of BAC vectors contained in the BAC-based DNA library I-1945 :

Rv101; Rv102; Rv103; Rv104; Rv105; Rv106; Rv107; Rv108; Rv109; Rv10; Rv110; Rv111; Rv112; Rv113; Rv114; Rv115; Rv116; Rv117; Rv118; Rv119; Rv11; Rv120; Rv121; Rv122; Rv123; Rv124; Rv126; Rv127; Rv128; Rv129; Rv130; Rv132; Rv134; Rv135; Rv136; Rv137; Rv138; Rv139; Rv13; Rv140; Rv141; Rv142; Rv143; Rv144; Rv145; Rv146; Rv147; Rv148; Rv149; Rv14; Rv150; Rv151; Rv152; Rv153; Rv154; Rv155; Rv156; Rv157; Rv159; Rv15; Rv160; Rv161; Rv162; Rv163; Rv164; Rv165; Rv166; Rv167; Rv169; Rv16; Rv170; Rv171; Rv172; Rv173; Rv174; Rv175; Rv176; Rv177; Rv178; Rv179; Rv17; Rv180; Rv181; Rv182; Rv183; Rv184; Rv185; Rv186; Rv187; Rv188; Rv18; Rv190; Rv191; Rv192; Rv193; Rv194; Rv195; Rv196; Rv19; Rv1; Rv201; Rv204; Rv205; Rv207; Rv209; Rv20; Rv214; Rv215; Rv217; Rv218; Rv219; Rv21; Rv220; Rv221; Rv222; Rv223; Rv224; Rv225; Rv226; Rv227; Rv228; Rv229; Rv22; Rv230; Rv231; Rv232; Rv233; Rv234; Rv235; Rv237; Rv240; Rv241; Rv243; Rv244; Rv245; Rv246; Rv247; Rv249; Rv24; Rv251; Rv252; Rv253; Rv254; Rv255; Rv257; Rv258; Rv259; Rv25; Rv260; Rv261; Rv262; Rv263; Rv264; Rv265; Rv266; Rv267; Rv268; Rv269; Rv26; Rv270; Rv271; Rv272; Rv273; Rv274; Rv275; Rv276; Rv277; Rv278; Rv279; Rv27; Rv280; Rv281; Rv282; Rv283; Rv284; Rv285; Rv286; Rv287; Rv288; Rv289; Rv28; Rv290; Rv291; Rv292; Rv293; Rv294; Rv295; Rv296; Rv29; Rv2; Rv301;

Rv302; Rv303; Rv304; Rv306; Rv307; Rv308; Rv309; Rv30; Rv310; Rv311; Rv312; Rv313; Rv314; Rv315; Rv316; Rv317; Rv318; Rv319; Rv31; Rv32; Rv322; Rv327; Rv328; Rv329; Rv32; Rv330; Rv331; Rv333; Rv334; Rv335; Rv336; Rv337; Rv338; Rv339; Rv33; Rv340; Rv341; Rv343; Rv344; Rv346; Rv347; Rv348; Rv349; Rv34; Rv350; Rv351; Rv352; Rv353; Rv354; Rv355; Rv356; Rv357; Rv358; Rv359; Rv35; Rv360; Rv361; Rv363; Rv364; Rv365; Rv366; Rv367; Rv368; Rv369; Rv36; Rv370; Rv371; Rv373; Rv374; Rv375; Rv376; Rv377; Rv378; Rv379; Rv37; Rv381; Rv382; Rv383; Rv384; Rv385; Rv386; Rv387; Rv388; Rv389; Rv38; Rv390; Rv391; Rv392; Rv393; Rv396; Rv39; Rv3; Rv40; Rv412; Rv413; Rv414; Rv415; Rv416; Rv417; Rv418; Rv419; Rv41; Rv42; Rv43; Rv44; Rv45; Rv46; Rv47; Rv48; Rv49; Rv4; Rv50; Rv51; Rv52; Rv53; Rv54; Rv55; Rv56; Rv57; Rv58; Rv59; Rv5; Rv60; Rv61; Rv62; Rv63; Rv64; Rv65; Rv66; Rv67; Rv68; Rv69; Rv6; Rv70; Rv71; Rv72; Rv73; Rv74; Rv75; Rv76; Rv77; Rv78; Rv79; Rv7; Rv80; Rv81; Rv82; Rv83; Rv84; Rv85; Rv86; Rv87; Rv88; Rv89; Rv8; Rv90; Rv91; Rv92; Rv94; Rv95; Rv96; Rv9.

The end sequences of the polynucleotide inserts of each of the above clones corresponding respectively to the sequences adjacent to the T7 promoter and to the Sp6 promoter on the BAC vector are shown in Table 3.

It has been shown by the inventors that the minimal overlapping set of BAC vectors of the BAC-based DNA library I-1945 contains 68 unique BAC clones and practically spans almost the whole H37Rv chromosome with the exception of a single gap of approximately 150 kb.

More specifically, a recombinant BAC vector of interest is choosen among the following set or group of BAC vectors from the BAC-based DNA library I-1945, the location of which vector DNA inserts on the chromosome of *M. tuberculosis* is shown in Figure 3 :

Rv234; Rv351; Rv166; Rv35; Rv415; Rv404; Rv209; Rv272; Rv30; Rv228; Rv233; Rb38; Rv280; Rv177; Rv48; Rv374; Rv151; Rv238; Rv156; Rv92; Rv3; Rv403; Rv322; Rv243; Rv330; Rv285; Rv233; Rv219; Rv416; Rv67; Rv222; Rv149; Rv279; Rv87; Rv273; Rv266; Rv25; Rv136; Rv414; Rv13; Rv289; Rv60; Rv104; Rv5; Rv165; Rv215; Rv329; Rv240; Rv19; Rv74; Rv411; Rv167; Rv56; Rv80; Rv164; Rv59; Rv313; Rv265; Rv308; Rv220; Rv258; Rv339; Rv121; Rv419; Rv418; Rv45; Rv217; Rv134; Rv17; Rv103; Rv21; Rv22; Rv2; Rv270; Rv267; Rv174; Rv257; Rv44; Rv71; Rv7; Rv27; Rv191; Rv230; Rv128; Rv407;

Rv106; Rv39; Rv255; Rv74; Rv355; Rv268; Rv58; Rv173; Rv264; Rv417; Rv401; Rv144; Rv302; Rv81; Rv163; Rv281; Rv221; Rv420; Rv175; Rv86; Rv412; Rv73; Rv269; Rv214; Rv287; Rv42; Rv143.

The polynucleotides disclosed in Table 3 may be used as probes in order to select a given clone of the BAC DNA library I-1945 for further use.

The invention also provides for a BAC-based *Mycobacterium bovis* strain Pasteur genomic DNA library that has been deposited in the Collection Nationale de Cultures de Microorganismes on June 30, 1998 under the accession number I-2049.

A further object of the invention consists in a recombinant BAC vector which is chosen among the group consisting of the recombinant BAC vectors belonging to the BAC-based DNA library I-2049. This DNA library contains approximately 1600 clones. The average insert size is estimated to be ~80 kb.

Generally, a recombinant BAC vector of interest may be chosen among the following set or group of BAC vectors contained in the BAC-based DNA library I-2049 :

X0001; X0002; X0003; X0004; X0006; X0007; X0008; X0009; X0010; X0012; X0013; X0014; X0015; X0016; X0017; X0018; X0019; X0020; X0021; X0175.

The end sequences of the polynucleotide inserts of each of the above clones corresponding respectively to the sequences adjacent to the T7 promoter and to the Sp6 promoter on the BAC vector are shown in Table 4.

The polynucleotides disclosed in Table 4 may be used as probes in order to select a given clone of the BAC DNA library I-2049 for further use.

Are also part of the invention the polynucleotide inserts that are contained in the above described BAC vectors, that are useful as primers or probes.

These polynucleotides and nucleic acid fragments may be used as primers for use in amplification reactions, or as nucleic probes.

PCR is described in the US patent N° 4,683,202. The amplified fragments may be identified by an agarose or a polyacrylamide gel electrophoresis, or by a capillary electrophoresis or alternatively by a chromatography technique (gel filtration, hydrophobic chromatography or ion exchange chromatography). The specificity of the amplification may be ensured by a molecular hybridization using, for example, one of the initial primers as nucleic probes.

Amplified nucleotide fragments are used as probes in hybridization reactions in order to detect the presence of one polynucleotide according to the

present invention or in order to detect mutations in the genome of the given mycobacterium of interest, specifically a mycobacterium belonging to the *Mycobacterium tuberculosis* complex and more specifically *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG.

Are also part of the present invention the amplified nucleic fragments (« amplicons ») defined herein above.

These probes and amplicons may be radioactively or non-radioactively labeled, using for example enzymes or fluorescent compounds.

Other techniques related to nucleic acid amplification may also be used and are generally preferred to the PCR technique.

The Strand Displacement Amplification (SDA) technique (Walker et al., 1992) is an isothermal amplification technique based on the ability of a restriction enzyme to cleave one of the strands at his recogntion site (which is under a hemiphosphorothioate form) and on the property of a DNA polymerase to initiate the synthesis of a new strand from the 3'OH end generated by the restriction enzyme and on the property of this DNA polymerase to displace the previously synthesized strand being localized downstream. The SDA method comprises two main steps :

a) The synthesis, in the presence of dCTP-alpha-S, of DNA molecules that are flanked by the restriction sites that may be cleaved by an appropriate enzyme.

b) The exponential amplification of these DNA molecules modified as such, by ezyme cleavage, strand displacement and copying of the displaced strands. The steps of cleavage, strand displacement and copy are repeated a sufficient number of times in order to obtain an accurate sensitivity of the assay.

The SDA technique was initially realized using the restriction endonuclease HincII but is now generally practised with an endonuclease from *Bacillus stearothermophilus* (BSOBI) and a fragment of a DNA polymerase which is devoid of any 5'→3'exonuclease activity isolated from *Bacilllus cladotenax* (exo- Bca) [=exo-minus-Bca]. Both enzymes are able to operate at 60°C and the system is now optimized in order to allow the use of dUTP and the decontamination by UDG. When unsing this technique, as described by Spargo et al. in 1996, the doubling time of the target DNA is of 26 seconds and the amplification rate is of 10^{10} after an incubation time of 15 min at 60°C.

The SDA amplification technique is more easy to perform than PCR (a single thermostated waterbath device is necessary) and is faster thant the other amplification methods.

Thus, another object of the present invention consists in using the nucleic acid fragments according to the invention (primers) in a method of DNA or RNA amplification according to the SDA technique. For performing SDA, two pairs of primers are used : a pair of external primers (B1, B2) consisting of a sequence specific for the target polynucleotide of interest and a pair of internal primers (S1, S2) consisting of a fusion oligonucleotide carrying a site that is recognized by a restriction endonuclease, for exemple the enzyme BSOBI.

The operating conditions to perform SDA with such primers are described in Spargo et al, 1996.

The polynucleotides of the invention and their above described fragments, especially the primers according to the invention, are useful as technical means for performing different target nucleic acid amplification methods such as :

- TAS (Transcription-based Amplification System), described by Kwoh et al. in 1989.
- SR (Self-Sustained Sequence Replication), described by Guatelli et al.in 1990.
- NASBA (Nucleic acid Sequence Based Amplification), described by Kievitis et al. in 1991.
- TMA (Transcription Mediated Amplification).

The polynucleotides according to the invention are also useful as technical means for performing methods for amplification or modification of a nucleic acid used as a probe , such as :

- LCR (Ligase Chain Reaction), described by Landegren et al. in 1988 and improved by Barany et al. in 1991 who employ a thermostable ligase.
- RCR (Repair Chain Reaction) described by Segev et al. in 1992.
- CPR (Cycling Probe Reaction), described by Duck et al. in 1990.
- Q-beta replicase reaction, described by Miele et al. in 1983 and improved by Chu et al. in 1986, Lizardi et al. in 1988 and by Burg et al. and Stone et al. in 1996.

When the target polynucleotide to be detected is a RNA, for example a mRNA, a reverse transcriptase enzyme will be used before the amplification reaction in order to obtain a cDNA from the RNA contained in the biological sample. The generated cDNA is subsequently used as the nucleic acid target for

the primers or the probes used in an amplification process or a detection process according to the present invention.

The non-labeled polynucleotides or oligonucleotides of the invention may be directly used as probes. Nevertheless, the polynucleotides or oligonucleotides are generally labeled with a radioactive element (^{32}P, ^{35}S, ^{3}H, ^{125}I) or by a non-isotopic molecule (for example, biotin, acetylaminofluorene, digoxigenin, 5-bromodesoxyuridin, fluorescein) in order to generate probes that are useful for numerous applications.

Examples of non-radioactive labeling of nucleic acid fragments are described in the french patent N° FR-7810975 or by Urdea et al. or Sanchez-Pescador et al., 1988.

In the latter case, other labeling techniques may be also used such as those described in the french patents FR-2 422 956 and 2 518 755. The hybridization step may be performed in different ways (Matthews et al., 1988). The more general method consists of immobilizing the nucleic acid that has been extracted from the biological sample onto a substrate (nitrocellulose, nylon, polystyrene) and then to incubate, in defined conditions, the target nucleic acid with the probe. Subsequently to the hybridization step, the excess amount of the specific probe is discarded and the hybrid molecules formed are detected by an appropriate method (radioactivity, fluorescence or enzyme activity measurement).

Advantageously, the probes according to the present invention may have structural characteristics such that they allow the signal amplification, such structural characteristics being, for example, branched DNA probes as those described by Urdea et al. in 1991 or in the European patent N° EP-0 225 807 (Chiron).

In another advantageous embodiment of the probes according to the present invention, the latters may be used as « capture probes », and are for this purpose immobilized on a substrate in order to capture the target nucleic acid contained in a biological sample. The captured target nucleic acid is subsequently detected with a second probe which recognizes a sequence of the target nucleic acid which is different from the sequence recognized by the capture probe.

The oligonucleotide probes according to the present invention may also be used in a detection device comprising a matrix library of probes immobilized on a substrate, the sequence of each probe of a given length being localized in a shift of one or several bases, one from the other, each probe of the matrix library thus

being complementary to a distinct sequence of the target nucleic acid. Optionally, the substrate of the matrix may be a material able to act as an electron donor, the detection of the matrix poisitons in which an hybridization has occurred being subsequently determined by an electronic device. Such matrix libraries of probes and methods of specific detection of a targer nucleic acid is described in the European patent application N° EP-0 713 016 (Affymax technologies) and also in the US patent N° US-5,202,231 (Drmanac).

Since almost the whole length of a mycobacterial chromososme is covered by a BAC-based genomic DNA libraries according to the present invention (i.e. 97% of the *M. tuberculosis* chromosome is covered by the BAC library I-1945), these DNA libraries will play an important role in a plurality of post-genomic applications, such as in mycobacterial gene expression studies where the canonical set of BACs could be used as a matrix for hybridization studies. Probing such matrices with cDNA probes prepared from total mRNA will uncover genetic loci induced or repressed under different physiological conditions (Chuang et al., 1993; Trieselmann et al., 1992). As such, the H37Rv BAC library represents a fundamental resource for present and future genomics investigations.

The BAC vectors or the polynucleotide inserts contained therein may be directly used as probes, for example when immobilized on a substrate such as described herein before.

The BAC vectors or their polynucleotide inserts may be directly asdorbed on a nitrocellulose membrane, at predetermined locations on which one or several polynucleotides to be tested are then put to hybridize therewith.

Preferably, a collection of BAC vectors that spans the whole genome of the mycobacterium under testing will be immobilized, such as, for example, the set of 68 BAC vectors of the I-1945 DNA library that is described elsewhere in the specification and shown in Figure 3.

The immobilization and hybridization steps may be performed as described in the present Materials and Methods Section.

As another illustrative embodiment of the use of the BAC vectors of the invention as polynucleotide probes, these vectors may be useful to perform a transcriptional activity analysis of mycobacteria growing in different environmental conditions, for example under conditions in which a stress response is expected, as it is the case at an elevated temperature, for example 40°C.

In this specific embodiment of the invention, Genescreen membranes may be used to immobilize the restriction endonuclease digests (*Hind*III digests for the BAC DNA library I-1945) of the BAC vectors by tranfer from a gel (Trieselmann et al., 1992).

Alternatively, the BAC vectors may be immobilized for dot blot experiments as follows. First, the DNA concentration of each BAC clone is determined by hybridization of blots of clone DNAs and of a BAC vector concentration standard with a BAC vector specific DNA probe. Hybridization is quantified by the Betascope 603 blot analyzer (Betagen Corp.), which colects beta particles directly from the blot with high efficiency. Then, 0.5 μg of each clone DNA is incubated in 0.25 M NaOH and 10 mM EDTA at 65°C for 60 min to denature the DNA and degrade residual RNA contaminants. By using a manifold filtration system (21 by 21 wells), each clone DNA is blotted onto a GeneScreen Plus nylon membrane in the alkaline solution. After neutralization, the blots are baked at 85°C for 2 h under vacuum. Positive and negative controls are added when necessary. In order to perform this procedure, it may be referred to the article of Chuang et al. (1993).

For RNA extractions, cells grown in a suitable volume of culture medium may, for example, be immediately mixed with an equal volume of crushed ice at -70°C and spun at 4°C in a 50 ml centrifugation tube. The cell pellet is then suspended in 0.6 ml of ice-cold buffer (10 mM KCl, 5 mM MgCl, 10 mM Tris; pH 7.4) and then immediately added to 0.6 ml of hot lysis buffer (0.4 M NaCl, 40 mM EDTA, 1% beta-mercaptoethanol, 1% SDS, 20 mM Tris; pH 7.4) containing 100 μl of water saturated phenol. This mixture is incubated in a boiling water bath for 40 s. The debris are removed by centrifugation. The supernatant is extracted with phenol-chloroform five times, ethanol precipitated, and dried. The dried RNA pellet is dissolved in water before use.

Then labeled total cDNA may be prepared by the following method. The reaction mixture contains 15 μg of the previously prepared total RNA, 5 μg of pd(N_6) (random hexamers from Pharmacia Inc.), 0.5 mM dATP, 0.5 mM dGTP and 0.5mM DTTP, 5μM dCTP, 100 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol), 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 40 mM Kcl, 0.5 U of avian myeloblastosis virus reverse transcriptase (Life Science Inc.) in a total volume of 50 μl. The reaction is allowed to continue overnight at room temperature. EDTA and NaOH are then added to final concentrations of 50 mM and 0.25 M, respectively, and

the mixture is incubated at 65°C for 30 min to degrade the RNA templates. The cDNA is then ready to use after neutralization by adding Hcl and Tris buffer.

The hybridization step may be performed as described by Chuang et al. (1993) and briefly disclosed hereinafter. The DNA dot blot is hybridized to ^{32}P-labeled total cDNA in a solution containing 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% sodium Pp_i, 0.1% bovine serum albumin, 0.5% SDS, 100 mM NaCl, and 0.1 mM sodium citrate, pH 7.2, at 65°C for 2 days and then washed with a solution containing 0.1% SDS, 100 mM NaCl, and 10 mM Na-citrate, pH 7.2. The same dot blot is used for hybridization with both control and experimental cDNAs, with an alkaline probe stripping procedure (soaked twice in 0.25M NaOH-0.75 M NaCl at room temperature, 30 min each, neutralized, and completely dried at 65°C for at least 30 min) between the two hybridizations. Quantification may be done with the Betascope 603 blot analyzer (Betagen Corp.).

As it flows from the above technical teachings, another object of the invention consists in a method for detecting the presence of mycobateria in a biological sample comprising the steps of :

a) bringing into contact the recombinant BAC vector or a purified polynucleotide according to the invention with a biological sample ;

b) detecting the hybrid nucleic acid molecule formed between said purified polynucleotide and the nucleic acid molecules contained within the biological sample.

The invention further deals with a method for detecting the presence of mycobacteria in a biological sample comprising the steps of :

a) bringing into contact the recombinant BAC vector or a purified polynucleotide according to the invention that has been immobilized onto a substrate with a biological sample ;

b) bringing into contact the hybrid nucleic acid molecule formed between said purified polynucleotide and the nucleic acid contained in the biological sample with a labeled recombinant BAC vector or a polynucleotide according to the invention, provided that said polynucleotide and polynucleotide of step a) have non-overlapping sequences.

Another object of the invention consists in a method for detecting the presence of mycobacteria in a biological sample comprising the steps of :

a) bringing into contact the nucleic acid molecules contained in the biological sample with a pair of primers according to the invention;

b) amplifying said nucleic acid molecules;

c) detecting the nucleic acid fragments that have been amplified, for example by gel electrophoresis or with a labeled polynucleotide according to the invention.

In one specific embodiment of the above detection and/or amplification methods, said methods comprise an additional step wherein before step a), the nucleic acid molecules of the biological sample have been made available to a hybridization reaction.

In another specific embodiment of the above detection methods, said methods comprise an additional step, wherein, before the detection step, the nucleic acid molecules that are not hybridized with the immobilized purified polynucleotide are removed.

Also part of the invention is a kit for detecting mycobacteria in a biological sample comprising :

a) a recombinant BAC vector or a purified polynucleotide according to the invention;

b) reagents necessary to perform a nucleic acid hybridization reaction.

The invention also pertains to a kit for detecting a mycobacteria in a biological sample comprising :

a) a recombinant BAC vector or a purified polynucleotide according to the invention that is immobilized onto a substrate;

b) reagents necessary to perform a nucleic acid hybridization reaction;

c) a purified polynucleotide according to the invention which is radioactively or non-radioactively labeled, provided that said polynucleotide and the polynucleotide of step a) have non-overlapping sequences.

Moreover, the invention provides for a kit for detecting mycobacteria in a biological sample comprising :

a) a pair of purified primers according to the invention;

b) reagents necessary to perform a nucleic acid amplification reaction;

c) optionally, a purified polynucleotide according to the invention useful as a probe.

The invention embraces also a method for detecting the presence of a genomic DNA, a cDNA or a mRNA of mycobacteria in a biological sample, comprising the steps of :

a) bringing into contact the biological sample with a plurality of BAC vectors according to the invention or purified polynucleotides according to the invention, that are immobilized on a substrate;

b) detecting the hybrid complexes formed.

The invention also provides a kit for detecting the presence of genomic DNA, cDNA or mRNA of a mycobacterium in a biological sample, comprising :

a) a substrate on which a plurality of BAC vectors according to the invention or purified polynucleotides according to the invention have been immobilized;

b) optionally, the reagents necessary to perform the hybridization reaction.

Additionally, the recombinant BAC vectors according to the invention and the polynucleotide inserts contained therein may be used for performing detection methods based on « molecular combing ». Said methods consist in methods for aligning macromolecules, especially DNA and are applied to processes for detecting, for measuring intramolecular distance, for separating and/or for assaying a macromolecule, especially DNA in a sample.

These « molecular combing » methods are simple methods, where the triple line S/A/B (meniscus) resulting form the contact between a solvent A and the surface S and a medium B is caused to move on the said surface S, the said macromolecules (i.e. DNA) having a part, especially an end, anchored on the surface S, the other part, especially the other end, being in solution in the solvent A. These methods are particularly fully described in the PCT Application n° PCT/FR 95/00165 files on February 11, 1994 (Bensimon et al.).

When performing the « molecular combing » method with the recombinant BAC vectors according to the inventions or their polynucleotide inserts, the latters may be immobilized (« anchored ») on a suitable substrate and aligned as described in the PCT Application n° PCT/FR 95/00165, the whole teachings of this PCT Application being herien incorporated by reference. Then, polynucleotides to be tested, preferably under the form of radioactively or non radioactively labeled polynucleotides, that may consist of fragments of genomic DNA, cDNA etc. are brought into contact with the previously aligned polynucleotides according to the present invention and then their hybridization position on the aligned DNA molecules is determined using any suitable means including a microscope or a suitable camera device.

Thus, the present invention is also directed to a method for the detection of the presence of a polynucleotide of mycobacterial origin in a biological sample

and/or for physical mapping of a polynucleotide on a genomic DNA, said method comprising :

a) aligning at least one polynucleotide contained in a recombinant BAC vector according to the invention on the surface of a substrate;

b) bringing into contact at least one polynucleotide to be tested with the substrate on which the at least one polynucleotide of step a) has been aligned;

c) detecting the presence and/or the location of the tested polynucleotide on the at least one aligned polynucleotide of step a).

The invention finally provides for a kit for performing the above method, comprising :

a) a substrate whose surface has at least one polynucleotide contained in a recombinant BAC vector according to the invention;

b) optionally, reagents necessary for labeling DNA;

c) optionnally, reagents necessary for performing a hybridization reaction.

In conclusion, it may be underlined that the alliance of such BAC-based approaches such as described in the present specification to the advances in comparative genomics by the availability of an increased number of complete genomes, and the rapid increase of well-characterized gene products in the public databases, will allow the one skilled in the art an exhaustive analysis of the mycobacterial genome.

MATERIALS AND METHODS

1. DNA-preparation. Preparation of *M. tuberculosis* H37Rv DNA in agarose plugs was conducted as previously described (Canard et al., 1989; Philipp et al., 1996b). Plugs were stored in 0.2 M EDTA at 4°C and washed 3 times in 0.1% Triton X-100 buffer prior to use.

2. BAC vector preparation. pBeloBAC11 was kindly provided by Dr. Shizuya, Department of Biology, California Institute of Technology (Pasadena, CA). The preparation followed the description of Woo et al., 1994 (Woo et al., 1994).

3. Partial digestion with *Hin*dIII. Partial digestion was carried out on plugs, each containing approximately 10 µg of high molecular weight DNA, after three one hour equilibration steps in 50 ml of *Hin*dIII 1X digestion buffer (Boehringer Mannheim, Mannheim, Germany) plus 0.1% Triton X-100. The buffer was then removed and replaced by 1ml/plug of ice-cold *Hin*dIII enzyme buffer containing 20 units of *Hin*dIII (Boehringer). After two hours incubation on ice, the plugs

were transferred to a 37°C water bath for 30 minutes. Digestions were stopped by adding 500 µl of 50 mM EDTA (pH 8.0).

4. Size selection. The partially digested DNA was subjected to contour-clamped homogenous electric field (CHEF) electrophoresis on a 1% agarose gel using a BioRad DR III apparatus (BioRad, Hercules, CA) in 1X TAE buffer at 13°C, with a ramp from 3 to 15 seconds at 6 V/cm for 16 hours. Agarose slices from 25 to 75 kb, 75 to 120 kb and 120 to 180 kb were excised from the gel and stored in TE at 4°C.

5. Ligation and transformation. Agarose-slices containing fractions from 25 to 75 kb, 75 to 120 kb and 120 to 180 kb were melted at 65°C for 10 minutes and digested with Gelase (Epicentre Technologies, Madison, WI), using 1 unit per 100 µl gel-slice. 25-100 ng of the size-selected DNA was then ligated to 10 ng of *Hin*dIII digested, dephosphorylated pBeloBAC11 in a 1:10 molar ratio using 10 units of T4 DNA ligase (New England Biolabs, Beverly, MA) at 16°C for 20 hours. Ligation mixtures were heated at 65°C for 15 minutes, then drop-dialysed against TE using Millipore VS 0.025 mM membranes (Millipore, Bedford, MA). Fresh electrocompetent *E. coli* DH10B cells (Sheng et al., 1995) were harvested from 200 ml of a mid-log (OD_{550}=0.5) culture grown in SOB medium. Cells were washed three times in ice-cold water, and finally resuspended in ice-cold water to a cell density of 10^{11} cells/ml (OD_{550}=150). 1 µl of the ligation-mix was used for electroporation of 30 µl of electrocompetent DH10B *E. coli* using a Eurogentec Easyject Plus electroporator (Eurogentec, Seraing, Belgium), with settings of 2.5 kV, 25 µF, and 99 Ω, in 2 mm wide electroporation cuvettes. After electroporation, cells were resuspended in 600 µl of SOC medium, allowed to recover for 45 minutes at 37°C with gentle shaking, and then plated on LB agar containing 12.5 µg/ml chloramphenicol (CM), 50 µg/ml X-gal, and 25 µg/ml IPTG. The plates were incubated overnight and recombinants (white colonies) were picked manually to 96 well plates. Each clone was inoculated 3 times (2 X 200 µl and 1 X 100 µl of 2YT/12.5 µg/ml CM per clone) and incubated overnight. One of the microtiter plates, containing 100 µl culture per well, was maintained as a master plate at - 80°C after 100 ml of 80% glycerol were added to each well, while minipreps (Sambrook et al., 1989) were prepared from the remaining two plates to check for the presence of inserts. Clones containing inserts were then designated "Rv" clones, repicked from the master plate to a second set of plates for storage of the library at -80°C.

6. Preparation of DNA for sizing, direct sequencing and comparative genomics. A modified Birnboim and Doly protocol (Birnboim et al., 1979) was used for extraction of plasmid DNA for sequencing purposes. Each Rv clone was inoculated into a 50 ml Falcon polypropylene tube containing 40 ml of 2YT medium with 12.5 μg/ml of CM and grown overnight at 37°C with shaking. Cells were harvested by centrifugation and stored at -20°C. The frozen pellet was resuspended in 4 ml of Solution A (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0) and 4 ml of freshly prepared solution B (0.2 M NaOH, 0.2% SDS) was then added. The solution was gently mixed and kept at room temperature for 5 minutes before adding 4 ml of ice-cold solution C (3M Sodium Acetate, pH 4.7). Tubes were kept on ice for 15 min, and centrifuged at 10,000 rpm for 15 min. After isopropanol precipitation, the DNA pellet was dissolved in 600 μl RNase solution (15 mM Tris HCl pH 8.0, 10 μg/ml RNase A). After 30 minutes at 37°C the DNA solution was extracted with chloroform:isoamylalcohol (24:1) and precipitated from the aqueous phase using isopropanol. The DNA pellet was then rinsed with 70% ethanol, air-dried and dissolved in 30 μl distilled water. In general, DNA prepared by this method was clean and concentrated enough to give good quality results by automatic sequencing (at least 300 bp of sequence). For a few DNA preparations, an additional polyethylene glycol (PEG) precipitation step was necessary, which was performed as follows. The 30 μl of DNA solution were diluted to 64μl, mixed gently and precipitated using 16 μl 4M NaCl and 80 μl of 13% PEG 8000. After 30 min on ice the tubes were centrifuged at 4°C, the pellet carefully rinsed with 70% ethanol, air-dried and diluted in 20 μl of distilled water.

7. Sizing of inserts. Insert sizes were determined by pulsed-field gel electrophoresis (PFGE) after cleavage with *Dra*I (Promega). 100-200 ng of DNA was *Dra*I-cleaved in 20 μl total reaction volume, following the manufacturer's recommendations, then loaded onto a 1% agarose gel and migrated using a pulse of 4 s for 15 h at 6.25 V/cm at 10°C on an LKB-Pharmacia CHEF apparatus. Mid-range and low-range PFGE markers (New England Biolabs) were used as size standards. Insert sizes were estimated after ethidium bromide staining of gels.

8. Direct sequencing. For each sequencing reaction 7 μl BAC DNA (300-500ng), 2 μl primer (2 μM), 8 μl reaction mix of the *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and 3 μl distilled water were used.

After 26 cycles (96°C for 30 sec; 56°C for 15 sec; 60°C for 4 min) in a thermocycler (MJ-research Inc., Watertown, MA) DNA was precipitated using 70 µl of 70% ethanol/0.5 mM $MgCl_2$, centrifuged, rinsed with 70% ethanol, dried and dissolved in 2 µl of formamide/EDTA buffer. SP6 and T7 samples of 32 BAC clones were loaded onto 64 lane, 6% polyacrylamide gels and electrophoresis was performed on a Model 373A automatic DNA sequencer (Applied Biosystems) for 12 to 16 hours. The sequences of oligonucleotides used as primers are shown in Table 1.

9. DOP-PCR. As an alternate procedure we used partially degenerate oligonucleotides in combination with vector-specific (SP6 or T7) primers to amplify insert ends of BAC clones, following a previously published protocol for P1 clones (Liu et al., 1995). The degenerate primers Deg2, Deg3, Deg4, Deg6 (Table 1) gave the best results for selected amplification of insert termini.

Table 1: Primers used for PCRs and sequencing

Vector specific Primers for DOP PCR- first amplification step:

SP6-BAC1: AGT TAG CTC ACT CAT TAG GCA

T7-BAC1 : GGA TGT GCT GCA AGG CGA TTA

Vector specific Primers (direct sequencing, nested primer for second PCR step)

SP6 Mid: AAA CAG CTA TGA CCA TGA TTA CGC CAA

T7-Belo2: TCC TCT AGA GTC GAC CTG CAG GCA

Degenerate Primers:

Deg2: TCT AGA NNN NNN TCC GGC

Deg3: TCT AGA NNN NNN GGG CCC

Deg4: CGT TTA AAN NNN NWA GGC CG

Deg6: GGT ACT AGT NNN NNW TCC GGC

Primers used for the amplification of *M. bovis* DNA in polymorphic chromosomal region of Rv58:

Primer 1: ACG ACC TCA TAT TCC GAA TCC C

Primer 2: GCA TCT GTT GAG TAC GCA CTT CC

10. Screening by pooled PCR. To identify particular clones in the library which could not be detected by random end-sequencing of the 400 BAC clones, PCR-screening of DNA pools was performed. Primers were designed for regions of the chromosome where no BAC coverage was appearent using cosmid-or H37Rv

whole genome shotgun sequences. Primers were designed to amplify approximately 400-500 bp. Ninety-six-well plates containing 200 µl 2YT/12.5 µg/ml CM per well were inoculated with 5 µl of -80°C glycerol stock cultures each from the master plates and incubated overnight. The 96 clones of each plate were pooled by taking 20 µl of culture from each well and this procedure was repeated for 31 plates. Pooled cultures were centrifuged, the pellets were resuspended in sterile water, boiled for 5 minutes, centrifuged and the supernatants kept for PCRs. As an initial screening step, the 31 pools of a total of 2976 BACs, representing about two thirds of the library were tested for the presence of a specific clone using appropriate PCR primers. PCR was performed using 10 µl of supernatant, 5 µl of assay buffer (100 mM b-mercaptoethanol, 600 mM Tris HCl (pH 8.8), 20 mM $MgCl_2$, 170 mM $(NH_4)_2SO_4$), 5 µl of Dimethylsulfoxide (DMSO), 5 µl of dNTPs (20 mM), 5 µl of water, 10 µl primer (2 µM), 10 µl inverse primer (2 µM) and 0.2 units of*Taq* DNA polymerase (Boehringer). 32 cycles of PCR (95°C for 30 s, 55°C for 1 min 30 s, 72°C for 2 min) were performed after an initial denaturation at 95°C for 1 min. An extension step at 72 °C for 5 min finished the PCR. If a pool of 96 clones yielded an appropriate PCR product (Fig. 1A), subpools were made to identify the specific clone. Subpools representative for lane A of a 96 well plate were made by pooling clones 1 to 12 from lane A into a separate tube. Subpools for lanes B to H were made in the same way. In addition, subpools of each of the 12 rows (containing 8 clones each) were made, so that for one 96 well plate, 20 subpools were obtained. PCR with these 20 subpools identified the specific clone (Fig. 1B, lower gel portion). If more than one specific clone was present among the 96 clones of one plate (Fig. 1B, upper gel portion), additional PCR reactions had to be performed with the possible candidates (data not shown).

11. Genomic comparisons. DNA from the BAC clone Rv58 was digested with the restriction endonucleases *Eco*RI and *Pvu*II, and resolved by agarose gel electrophoresis at low voltage overnight (1.5 V/cm). DNA was transferred via the method of Southern to nitrocellulose membranes (Hybond C extra, Amersham) following standard protocols (Sambrook et al., 1989), then fixed to the membranes at 80°C for 2 hours. The blot was hybridized with ^{32}P labelled total genomic DNA from *M. tuberculosis* H37Rv, *M. bovis* type strain (ATCC 19210) or *M. bovis* BCG Pasteur. Hybridization was performed at 37°C overnight in

50% formamide hybridization buffer as previously described (Philipp et al., 1996b). Results were interpreted from the autoradiograms.

12. Computer analysis. Sequence data from the automated sequencer ABI373A were transferred as binary data to a Digital Alpha 200 station or Sun SparcII station and analysed using TED, a sequence analysis program from the Staden software package (Dear et al., 1991). Proof-read sequences were compared using the BLAST programs (Altschul et al., 1990) to the *M. tuberculosis* H37Rv sequence databases of the Sanger Centre, containing the collected cosmid sequences (TB.dbs) and whole-genome shotgun reads (TB_shotgun_all.dbs) (http://www.sanger.ac.uk/). In addition, local databases containing 1520 cosmid end-sequences and the accumulating BAC end-sequences were used to determine the exact location of end-sequenced BACs on the physical and genetic map. MycDB (Bergh et al., 1994) and public databases (EMBL, Genbank) were also used to compare new sequences, but to a lesser extent. The organization of the open reading frames (ORFs) in the polymorphic region of clone Rv58 was determined using the DIANA software established at the Sanger Centre.

EXAMPLES

Example 1 : Construction of a pBeloBAC11 library of *M. tuberculosis* H37Rv.

Partial *Hin*dIII fragments of H37Rv DNA in the size range of 25 to 180 kb were ligated into pBeloBAC11 and electroporated into strain *E. coli* DH10B. While cloning of fractions I (25 to 75 kb) and II (75 to 120 kb) gave approximately 4 x 10^4 transformants (white colonies), cloning of fraction III (120 to 180 kb) repeatedly resulted in empty clones. Parallel cloning experiments using partial *Hin*dIII digests of human DNA resulted in stable inserts for all three fractions (data not shown), suggesting that the maximum size of large inserts in BAC clones is strongly dependent on the source of the DNA. Analysis of the clones for the presence of inserts revealed that 70 % of the clones had an insert of the appropriate size while the remaining 30% of white colonies represented empty or *lacZ'*-mutated clones. Size determination of randomly selected, *Dra*I-cleaved BACs via PFGE showed that the insert sizes ranged for the majority of the clones between 40 kb and 100 kb with an average size of 70 kb. Clones with inserts of appropriate size were designated with "Rv" numbers, recultured and stored at -80°C for further use.

Example 2 : Direct DNA sequence analysis of BACs.

To characterize the BAC clones, they were systematically subjected to insert termini sequencing. Two approaches, direct sequencing of BAC DNA and PCR with degenerate oligonucleotide primers (DOP), adapted to the high G+C content of mycobacterial DNA, were used. In a first screening phase, 50 BAC clones designated Rv1 to Rv50 were analysed using both methods in parallel. Except for two clones, where the sequences diverged significantly, the sequences obtained by the two methods only differed in length. Sequences obtained directly were on average about 350 bp long and for 95% of the clones both the SP6 and T7 end-sequences were obtained at the first attempt. Sequences obtained by DOP-PCR were mostly shorter than 300 bp. For 40% of the BACs we obtained only very short amplicons of 50 to 100 base pairs from one end. In two cases the sequence obtained with the DOP-PCR differed from the sequences obtained by direct sequencing, and in these cases *E.coli* or vector sequences were amplified (data not shown). Taking the advantages and disadvantages of both methods into account, we decided to use direct termini sequencing for the systematic determination of the SP6 and T7 end-sequences.

Example 3 : Representativity of the library.

After having determined the end-sequences of 400 BACs a certain redundancy was seen. The majority of clones were represented at least 3 to 4 times. Maximum redundancy was seen in the vicinity of the unique *rrn* operon, as 2.5 % of the clones carried identical fragments that bridge the cosmids Y50 and Y130 (Fig. 3, approximate position at 1440 kb). The majority of clones with identical inserts appeared as two variants, corresponding to both possible orientations of the *Hin*dIII fragment in pBeloBAC11. This suggests that the redundancy was not the result of amplification during library construction, but due to the limited number of possible combinations of partial *Hin*dIII fragments in the given size-range of 25 to 120 kb. To detect rare BAC clones, a pooled PCR protocol was used. Primers were designed on the basis of the existing cosmid sequences and used to screen 31 pools of 96 BAC clones. When positive PCR products of the correct size were obtained, smaller subpools (of 8 or 12 clones each) of the corresponding pool were subsequently used to identify the corresponding clone (Figs. 1A and 1B). With this approach 20 additional BACs (Rv401-Rv420) were found for the regions where no BACs were found with the initial systematic sequencing approach. The end-sequences of these BACs

(Rv401-420) were determined by direct sequencing, which confirmed the predicted location of the clones on the chromosome. A 97% coverage of the genome of H37Rv with BAC clones was obtained. Only one region of ~ 150 kb was apparently not represented in the BAC library as screening of all pools with several sets of specific primers did not reveal the corresponding clone. This was probably due to the fact that *Hin*dIII fragments of mycobacterial DNA larger than 110 kb are very difficult to establish in *E. coli* and that a *Hin*dIII fragment of ~120 kb is present in this region of the chromosome (data not shown).

Example 4 : Establishing a BAC map.

Using all end-sequence and shotgun-sequence data from the H37Rv genome sequencing project, most of the BAC clones could then be localized by sequence comparison on the integrated map of the chromosome of *M. tuberculosis* strain H37Rv (Philipp et al., 1996b) and an ordered physical map of the BAC-clones was established. PCR with primers from the termini sequences of selected BACs were used for chromosomal walking and confirmation of overlapping BACs (data not shown). The correct order of BACs on the map was also confirmed more recently, using 40,000 whole genome shotgun reads established at the Sanger Centre. In addition, pulsed-field gel electrophoresis of *Dra*I digests of selected BACs was performed (Fig. 2) in order to see if the approximate fragment size and the presence or absence of *Dra*I cleavage sites in the insert were consistent with the location of the BACs on the physical map (Fig. 3). Comparison of the sequence-based BAC-map with the physical and genetic map, established by PFGE and hybridization experiments (Philipp et al., 1996b), showed that the two maps were in good agreement. The positions of 8 genetic markers previously shown on the physical and genetic map were directly confirmed by BAC-end-sequence data (**Table 2**, Fig. 3). The position of 43 from 47 Y-clones (91%) shown on the physical and genetic map, which were later shotgun sequenced, was confirmed by the BAC end-sequences and shotgun sequence data. Four clones (Y63, Y180, Y251, and Y253) were located to different positions than previously thought and this was found to be due to book keeping errors or to chimeric inserts. Their present approximate location relative to the *oriC* is shown in Figure 3: Y63 at 380 kb, Y63A at 2300 kb, Y180 at 2160 kb, Y251 at 100 kb, and Y253 at 2700 kb. A total of 48 BACs, covering regions of the chromosome, not represented by cosmids were then shotgun sequenced (Cole et al., 1997), and these are squared in Fig. 3. No chimeric BACs

were found, which is consistent with the observations of other research groups for other BAC libraries (Cai et al., 1995; Zimmer et al., 1997). The absence of chimeric BACs was of particular importance for the correct assembly of the *M. tuberculosis* H37Rv sequence. The exact position of the BAC termini sequences on the chromosome will be available via the world wide web (http://www.pasteur.fr/MycDB).

Table 2 : Identities of genetic markers previously shown on the integrated and genetic map of H37Rv (Phlipp et al., 1996b) wich showed perfect sequence homology with BAC ens sequences.

Locus	BAC end sequence	Description of genetic marker	Organism	GenBank Accession n°
apa	Rv163SP6	Secreted alanine-proline-rich antigen	*M. tuberculosis*	X80268
dnaJ, dnaK	Rv164T7	DnaJ hsp	*M. leprae*	M95576
fop-A	Rv136T7	Fibronectin binding protein	*M. tuberculosis*	M27016
polA	Rv401T7	DNA polymerase I	*M. tuberculosis*	L11920
ponA	Rv273T7	Penicillin binding protein	*M. leprae*	S82044
pstC	Rv103T7	Putative phosphate transport receptor	*M. tuberculosis*	Z48057
recA	Rv415SP6	Homologous recombination	*M. tuberculosis*	X58485
wag9	Rv35SP6	35-kDa antigen	*M. tuberculosis*	M69187

Example 5 : Repetitive end-sequences.

Repetitive sequences can seriously confound mapping and sequence assembly. In the case of the BAC end-sequences, no particular problems with repetitive sequences were observed. Although nine clones with one end in an IS*1081* (Collins et al., 1991) sequence were identified, it was possible to correctly locate their position on the map using the sequence of the second terminus. Moreover, these BACs were used to determine the exact locations of IS*1081* sequences on the map. Five copies of this insertion sequence, which

harbors a *Hin*dIII cleavage site, were mapped on the previous physical and genetic map. In contrast, BAC end-sequence data revealed an additional copy of IS*1081* on the *M. tuberculosis* H37Rv chromosome. The additional copy was identified by six clones (Rv27, Rv118, Rv142, Rv160, Rv190, Rv371) which harbored an identical fragment linking Y50 to I364 (Fig. 3, at ~ 1380 kb). This copy of IS*1081* was not found by previous hybridization experiments probably because it is located near another copy of IS*1081*, localized on the same *Dra*I fragment Z7 and *Asn*I fragment U (Fig. 3, at ~ 1140 kb). Furthermore, the position of a copy of IS*1081* previously shown in *Dra*I fragment Y1 (Fig. 3, at ~ 1840 kb) had to be changed to the region of Y349 (Fig. 3, at ~ 3340 kb) according to the end-sequences of BAC Rv223. The positions of the four other IS*1081* copies were confirmed by the sequence data and therefore remained unchanged. In total 6 copies of IS*1081* were identified in the H37Rv genome in agreement with the findings of others (Collins et al., 1991).

In addition, a sequence of 1165 bp in length containing a *Hin*dIII site was found in two copies in the genome of H37Rv in different regions. The end-sequences of BAC clones Rv48 and Rv374, covering cosmid Y164, as well as Rv419 and Rv45, that cover cosmid Y92, had perfect identity with the corresponding parts of this 1165 bp sequence (Fig. 3, at ~ 3480 kb and ~ 900 kb). Analysis of the sequence did not reveal any homology with insertion sequences or other repetitive elements. However, as each of the two locations showed appropriate BAC coverage, chimerism of the sequenced cosmids Y164 and Y92 can be ruled out as the probable cause.

Example 6 : Using BAC clones in comparative genomics.

The minimal overlapping set of BAC clones represents a powerful tool for comparative genomics. For example, with each BAC clone containing on average an insert of 70 kb, it should be possible to cover a 1Mb section of the chromosome with 15 BAC clones. Restriction digests of overlapping clones can then be blotted to membranes, and probed with radiolabelled total genomic DNA from, for example, *M. bovis* BCG Pasteur. Restriction fragments that fail to hybridize with the *M. bovis* BCG Pasteur DNA must be absent from its genome, hence identifying polymorphic regions between *M. bovis* BCG Pasteur and *M. tuberculosis* H37Rv. The results of such an analysis with clone Rv58 (Fig. 3, at ~1680 kb) are shown here. This clone covers a previously described polymorphic genomic region between *M. tuberculosis* and *M. bovis* BCG strains (Philipp et

al., 1996a). *Eco*RI and *Pvu*II digests from clone Rv58, fixed on nitrocellulose membranes, were hybridized with ^{32}P-labelled total genomic DNA from *M. tuberculosis* H37Rv, *M. bovis* (ATCC 19120), and *M. bovis* BCG Pasteur. Figures 4A and 4B present the results of this analysis, where it is clear that several restriction fragments from clone Rv58 failed to hybridize with genomic DNA from either *M. bovis* or *M. bovis* BCG Pasteur. On the basis of the various missing restriction fragments, a restriction map of the polymorphic region was established and compared to the H37Rv sequence data. The localization of the polymorphism could therefore be estimated, and appropriate oligonucleotide primers (Table 1) were selected for the amplification and sequencing of the corresponding region in *M. bovis*. The alignment of *M. bovis* and *M. tuberculosis* H37Rv sequences showed that 12,732 bp were absent from the chromosomal region of the *M. bovis* type strain and *M. bovis* BCG Pasteur strain. The G+C content of the polymorphic region is 62.3 mol%, which is the same as the average genome G+C content of the *M. tuberculosis* genome, hence indicating that this region is not a prophage or other such insertion. Subsequent PCR studies revealed that this segment was also absent from the Danish, Russian, and Glaxo substrains of *M. bovis* BCG, suggesting that this polymorphism can be used to distinguish *M. bovis* from *M. tuberculosis*. Analysis of this sequence showed that 11 putative open reading frames (ORFs) are present in *M. tuberculosis*, corresponding to ORFs MTCY277.28 to MTCY277.38 / accession number Z79701 -EMBL Nucleotide Sequence Data Library (Fig. 5). FASTA searches against the protein and nucleic acid databases revealed that the genes of this region may be involved in polysaccharide biosynthesis. Among these putative genes, the highest score was seen with ORF 6 (MTCY277.33), whose putative product shows a 51.9% identity with GDP-D-Mannose dehydratase from *Pseudomonas aeruginosa* (accession number U18320 - EMBL Nucleotide Sequence Data Library) in a 320 amino acid overlap. The novel *M. bovis* sequence of the polymorphic region was deposited under accession number AJ003103 in the EMBL Nucleotide Sequence Data Library.

As it appears from the teachings of the specification, the invention is not limited in scope to one or several of the above detailed embodiments; the present invention also embraces all the alternatives that can be performed by one skilled in the same technical field, without deviating from the subject or from the scope of the instant invention.

Table 3 : End-sequences of the polynucleotide inserts cloned in the named recombinant BAC vectors contained in the I-1945 *M. tuberculosis* H37Rv genomic DNA library.

RvXXXSP6 corresponds to the SP6 end-sequence of the clone RvXXX.

RvXXXT7 corresponds to the T7 end-sequence of the clone RvXXX.

RvXXXIS 1081 corresponds to a region located close to a copy of the IS1081 repetitive sequence (Insertion element).

The character « - » denotes an uncertain base residue.

```
Clone Rv101
::::::::::::::Rv101SP6.seq::::::::::::::
AATACTCAAGCTTGCCCAGCCGTCGATGACAAGAAATATGTCCGCAAAAGACTCAGCGGCCGACTTTGCTCGCAGCTG
GCGGTACCGCGCCACCGATTCTATGCCGTGGTCGCGGAAAAATGCCTCCCGAAATCGCACGGCCGACTCCAGTTCGGC
GAGCATCCGCGATGCCAGCTGCGGCTGCGCCCTGCCGGCCACGGCACCCACATGCGGCAGTTCGTCCACCTGGGCCAG
CGCCCCGCCGCCGAATTCCAAACAATAGAACTGCACCCGGCCCGCATCGTGGGTAACAGCCAACGCCATGATCAGCGT
CCGCAGCGCGGTTGACTTGCCCGTTTGCGGTGCACCTACGAACGCGACATTGCCTGCGGCCCCGGACAAGTCGATCGT
GCGCGGCACCCGTGACTGCTCTAACGGGCGATTGAAATTCCGAT

::::::::::::::Rv101T7.seq::::::::::::::
CCACCCGTGTAATTTGGGATGGGCAAAAAGGCGAAGCACCGCGTGGCCACGAACGCCGGGAGGGACAATCTCGGGCGG
TTAGGGCTTCTCGCGGGAAGGCCCGAACGTACGGCGTTTCAACACCTCGCGTCGCCCTCCGACCGCGAACATTCGGGG
ATGGCAGCAACCTGCTGGCACCCTGGCCGGGCGATGATCTGCAGCGTCGCCGCGGGTAGTCGCCGCCCGGGCGGCTAC
ACTCTGAAACGCGATGACCATCGATGTGTGGATGCAGCATCCCGACGCAACGGTTCCTACACCGCGATATGTTCGCCT
CGCTGCCCCGGTGGACCGGT


Clone Rv102
::::::::::::::Rv102SP6.seq::::::::::::::
AATACTCAAGCTTTCCGCCGATACCCGCCATGTCGCGCACATCCAGGACTTCTGGGGGGATCCGCTGACAGCGGCGGG
ATCCCAAAGTGCGGATGATCGGGCCGCCTACGTCGTGGTGTACCTCGTCGGTAACAACGAAACCGAAGCGTATGACTC
GGTCCACGCGGTGCGGCACATGGTGGACACCACACCGCCACCGCACGGGGTGAAGGCCTATGTCACCGGTCCGGCAGC
ACTCAATGCCGACCAGGCCGAGGGCGGAGACAAAAGTATCGCTAAGGTCACCGCGATCACCAACATGGTGATCGCAGC
AATGTTGCTAGTGATCTATCGCTCCGTAATTACCGCGGTTCT

::::::::::::::Rv102T7.seq::::::::::::::
GTGCCGTTCCAACCCGAATTGGCTTTCGGCGCCATCGGTGAGGACGGCGTGCGGGTGCTCAACGACGACGTCGTCCGC
GGGACACACCTCGATGCTGCCGCCATGGACGCGGTCGAACGCAAGCAGCTGATCGAGCTACAACGCCGCGCGGAACGC
TTCCGCCGCGGGCGTGACCGCATCCCGTTGACCGGGCGGATCGCGGTGATCGTCGATGACGGCATCGCCACCGGAGCG
ACGGCCAAGGCGGCGTGCCAGGTCGCCCGGGCGCACGGTGCGGACAAGGTGGTGCTGGCGGTCCCGATCGGCCCAGAC
GACATCGTGGCGAGATTCGCCGGGTACGCCGATGAGGTGGT


Clone Rv103
::::::::::::::Rv103SP6.seq::::::::::::::
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Clone Rv127
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Clone Rv128
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Clone Rv129
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Clone Rv130
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Clone Rv134
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Clone Rv135

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Clone Rv136

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Clone Rv137

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Clone Rv138

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Clone Rv139
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Clone Rv13
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Clone Rv140
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Clone Rv143

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Clone Rv146

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Clone Rv147

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ACCCACGACGGAAAGACGCAGATCGCCGTCTANCNTGTGTGCCGCGGATTATCAGGACTGACCTCCTGGCTGACCGGC
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Clone Rv148

:::::::::::::Rv148SP6.seq:::::::::::::

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GTCCACGCGGTGCGGCACATGGTGGACACCACACCGCCACCGCACGGGGTGAAGGCCTATGTCACCGGTCCGGCAGCA
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CAACGCCGCGCGGAACGCTTCCGCCGCGGGCGTGACCGCATCCCGTTGACCGGGCGGATCGCGGTGATCGTCGATGAC
GGCATCGCCACCGGAGCGACGGCCAAGGCGGCGTGCCAGGTCGCCCGGGCGCACGGTGCGGACAAGGTGGTGCTGGCG
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Clone Rv149

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GCCGGTCAGGGTGCCCTTCCAGTCCACGCCGCTGTGGTCGGCGAACCGCTTATCTTCAATCGAGACGATCGCCAGCTT
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Clone Rv14

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TTCCAGGTCCAGCGGGTGGCGCAGCAACGGCGCGAGCTCAACGACGTCAATCACGTTGTCGCTTTCTACGGTCACCGA
CCCGGTGACCGTAGTCGCCCGGTGCGCTCGGCCGAGAAGTTGCACCGCCACCACCGCGACACCGTCTTGCACGCGGAC
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Clone Rv15

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Clone Rv151

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GAAGACGGGGCCGGCTGGCTGGCGTTCTTCCGCGACCTGGTCGCCCGCGGCCTGTCCGGGGTCGCGCTGGTCACCAGC
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AGCGAATCCGAGAGCAGGAGGACGAGGTCACGAAGCTGCGCCAGCCGGTCGTACCGCTCAGGGCGGATGTCGCCGGTC
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Clone Rv152

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GACGGTGAACAGGTTGCGCTGGCCACCGCGGGTAGCCGCCGGCACTATGCCGATGCCCAGGCCGCGGATCACCGGTGC
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Clone Rv153

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CCGTTGTGCTGGTGGGTGTGCCGACGCCCGACATGCGCCTGGACATGCCGCTGGTCGACTTCTTCTCTCACGGCGGTG
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Clone Rv154

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Clone Rv155

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ATCATCACCACGCTCGACGTGGTTAGCGCCGGTCGAGCGATCCTCGGCATTGGAGCCGGTTGGTTTGAGCTGGAACAC
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Clone Rv156
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Clone Rv157
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Clone Rv159
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Clone Rv15
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Clone Rv160

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CCT

Clone Rv161

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Clone Rv162

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Clone Rv163

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CCT

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Clone Rv164
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Clone Rv165
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Clone Rv166
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Clone Rv167
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Clone Rv169
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Clone Rv170
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Clone Rv171

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Clone Rv173

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Clone Rv174

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Clone Rv175

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Clone Rv176
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Clone Rv178
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Clone Rv184
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Clone Rv207

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Clone Rv209

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Clone Rv223
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Clone Rv228
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Clone Rv229

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Clone Rv22

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Clone Rv230

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Clone Rv231

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Clone Rv23
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Clone Rv251
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Clone Rv259
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Clone Rv25
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Clone Rv270

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Clone Rv271
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Clone Rv272
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Clone Rv273
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Clone Rv274
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Clone Rv275
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Clone Rv276
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Clone Rv279
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Clone Rv27
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Clone Rv281
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Clone Rv289

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Clone Rv290

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Clone Rv291

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Clone Rv292

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Clone Rv293
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Clone Rv295
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Clone Rv296

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Clone Rv29

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Clone Rv301

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Clone Rv302
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Clone Rv303
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Clone Rv304
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Clone Rv306
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Clone Rv307

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Clone Rv308

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Clone Rv309

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Clone Rv30

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Clone Rv310
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Clone Rv311
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Clone Rv312
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Clone Rv313
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Clone Rv314
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Clone Rv315
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Clone Rv316
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Clone Rv317
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Clone Rv322
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Clone Rv329
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Clone Rv32

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Clone Rv330

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Clone Rv331

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Clone Rv333

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Clone Rv334

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Clone Rv335
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Clone Rv337
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Clone Rv339
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Clone Rv33
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Clone Rv341
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Clone Rv344

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Clone Rv346

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Clone Rv347

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Clone Rv348

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TGGTCGATCGAAGGGGGTCGGACCAGAACGGGCAAGCTACGGCCACCGGTGTTCGGGATCCTGCGTTACATCACCGAT
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Clone Rv418
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Clone Rv419
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Clone Rv41
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GGTGGCGGCCGCGCCGT

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Clone Rv42

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GCGCCACGTCTCCAACGCGCCACGCGCCATACCTGGTGCCAGTTGCTTGCGCATCCGGGTGTGCGCCGGCGGATCGGA
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Clone Rv43

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CACCAGCGACNTCTCCGGGGTTGACCGGGTGATCTCGGCGAAATCAGATACGCTGGCGCCGCGACGACCAGGCGTCGT
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CGTAGGCATTGGCCGTCACAACAGGCGACGCCCCGCGTACCACCGAGTCCACGGNGGTTGGGCGGTCTCCTCGGCCAA
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Clone Rv44

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:::::::::::::Rv44-2ndT7.seq:::::::::::::

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GAGGTCGTCGAGACCGAGCTGATCCTGGCAGATCTGCAAACCCTGGAGCGGGCCACGGGCCGGCTGGAGAANGAAGCN
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Clone Rv45

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CGTCGGCACGCATCACCCCGCCGTNTGCCGCCGAGTGCGCCTCGAGTTTGGCGGCCAGCTCTCCCCGGTAGAACTCTC
ACCGTTGGTCGCCGCGATCTTCTCTANCGTCGCCGCGTGGTCAGGAAAGGTAAACAGCTCACCGGGTTTCGGCGCTCG
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Clone Rv46
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Clone Rv47
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Clone Rv48
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Clone Rv49
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CCGGTCGACACAACGGAAGCGAAAGTGTCCGCGATGTTGATGAGCGTCGCCGGTTGTGGCGGCGGTGGCGGCGGTAGC
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Clone Rv4

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Clone Rv50

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Clone Rv51

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Clone Rv52

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Clone Rv53

:::::::::::::Rv53SP6.seq::::::::::::

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TCGCCGGTCCCCCGCCCACATACAGCGGAGGATAGGGCTTTGTCACCGGCTTCGGCCAGCAGTAGATCGGATCGAAGT
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Clone Rv54

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Clone Rv55

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Clone Rv56

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Clone Rv57
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Clone Rv58
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Clone Rv59
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Clone Rv5
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Clone Rv60
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Clone Rv61
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Clone Rv65
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Clone Rv66
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Clone Rv67
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Clone Rv69
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Clone Rv6
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Clone Rv72
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Clone Rv75
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Clone Rv77

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Clone Rv79

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Clone Rv7

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Clone Rv80

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Clone Rv81

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Clone Rv82

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Clone Rv83
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Clone Rv86
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GGCCANGGATCAAAGCCCGTG

Clone Rv87
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Clone Rv88
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CCAACNACACCCNCGTCTGGGTGTACTGCGGCAACNGCAAGCCGTCGGATCTGGGTGGCAACAACCTGCCGGCCAAGT
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TCGACTTCCCGG

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Clone Rv89
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GTNTTGCTCGGACTN

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NTCGGGCCNAGCANTCCCGGTGCNACCGNGGTGGGTCCGGCGATGGTNGGGTGTNCTCNNTACNGGAACGCCAGCGCN
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Clone Rv8

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Clone Rv90

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Clone Rv91

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Clone Rv92

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TGGCCTTCGGCAAGCNCNANANAACGCGACCCGGAGGTGTTGAACTAGCTTCGCCGCGTATTTACAAATTGCNTTATA
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Clone Rv94

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AACGCTNATCTTCAATCGAGACCATCGCCAGCTTCATCNTGTTGGCGATCTTGTCNNACGGCACCTCNAACCGGCGCT
NCTAGTACNCCACNCNATCNTGTTNCCTTCNCGTCNACATCCTCGATNCCNCNTGCACTTTCCCTCGANCNCCTGGGC
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ACNCCNCCTCNTGACNGCACCAGTGCNCTGCCCTTCTTGTGCACGTACCCGCNATCCTGGATGGTCTTGATGATCNAC
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Clone Rv95

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ATACCGGCAGCGCGAACATCNCCGCGGGCTGCAGCNTGAACGTCCAATACCANTCNAACAGTGTCCGCGCGTNAAAAC
CCGANCCGGCGGTCGCTTCNGTAATCAACGGCTCCTGCGCAACCAGCTGCAAGTCGCCGGTGCCACCGGCGTTGACGA
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Clone Rv96

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AGCATTTCTCCAGGCACCAGTTGAAGAGCGTTGATCAATGGCCTGTTCAAAAACAGTTCTCATCCGGATCTGACCTTT
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Clone Rv9

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ANTTTCAGGACCCGAATGCTCCGGGAAACATGTCACGGTAGGTCGGTATTCCGGCTACCGGCTGANCATTGAGCACGC
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GCGATTGGCCGGCGCGGCGCTGGTCGCCAAGGAAGACCCGTTCCGGTGCTGAT

Table 4 : End-sequences of the polynucleotide inserts cloned in the named recombinant BAC vectors contained in the I-2049 *M. bovis* strain Pasteur genomic DNA library.

RvXXXSP6 corresponds to the SP6 end-sequence of the clone RvXXX.

RvXXXT7 corresponds to the T7 end-sequence of the clone RvXXX.

RvXXXIS 1081 corresponds to a region located close to a copy of the IS1081 repetitive sequence (Insertion element).

The character « - » denotes an uncertain base residue.

```
Clone X0001
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TCGCGGTGGTCGG-CGGGTTGCGTGCCGCCTGCTGCGA-ATGCACCA-
TAAGCCCGAACCCACCGGCTTGGTGACCACCGCACGCTGCGTGTGGGGGTAACCACTCCGCGACCCCAAGGATGGT
CATTTCCAATGAACCGGCTGGACTTCGTCCA-A

::::::::::::::X0001T7.seq::::::::::::::
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AGCCGGAATGTCTGCGTAAAGAAGTTCCATGTCCGGGAAGTAGACCCGGTCGCCCTCCACGTGGTACTCCTTCGAGG
TCCGCTTCTCGCCGGATCCGATAAACACCGGCCCCAGGCACCGCAGCGTGAGTTCGAACGGCTTCAGGTAGGTGTTC
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TCGAAGATGCCTCCTTGGAAGGGGCGCGA


Clone X0002
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AACGACGCCAGTCCGCTACGTAACCCCTCCGCGACTGTCCATGGACAACAGCGCGTTCTCCACCGACCGGGCCCGGG
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AAAGACGCAGATCGCCGTCAAGCATGTGTGCCGCGGATTATCAGGACTGACCTCCTGGCTGACCGGCATGTTTGGTC
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AGGGTCCTGCCGGTGCCGGT


Clone X0003
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CTCTTTGAGTGGCGCCGTCGCCTGTGCCCCATCGGTGTTCATGACGAACGCTTCGAAAGACTTCCTCTTGTGAGCCG
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TTCTC
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ATTTCCAATGAACCGGCTGGACTTC-TCAACAA

Clone X0004
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Clone X0006
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CLAIMS

1. A method for isolating a polynucleotide of interest that is present in the genome of a mycobacterium strain and/or is expressed by said mycobacterium strain and that is absent or altered in the genome of a different mycobacterium strain and/or is not expressed in said different mycobacterium strain, said method comprising the use of at least one clone belonging to a genomic DNA library of a given mycobaterium strain, said DNA library being cloned in a bacterial artificial chromosome (BAC) vector.

2. The method according to claim 1, wherein the BAC-based DNA library has beeen constructed from genomic DNA of *Mycobacterium tuberculosis*.

3. The method according to claim 2, wherein the BAC-based DNA library has beeen constructed from genomic DNA of *Mycobacterium tuberculosis* strain H37Rv.

4. The method according to claim 3, wherein the BAC-based DNA library has been deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) on November 19, 1997 under the accession number I-1945.

5. The method according to claim 1, wherein the BAC-based DNA library has been constructed from genomic DNA of *Mycobacterium bovis*.

6. The method according to claim 5, wherein the BAC-based DNA library has been constructed from the genomic DNA of *Mycobacterium bovis* BCG strain Pasteur.

7. The method according to claim 6, wherein said DNA library contains approximatively 1600 clones and wherein the genomic DNA is cloned into a recombinant pBeloBAC11 vector with an average insert size of approximately 80 kb.

8. The method according to claim 6 or 7, wherein the at least one BAC-based DNA library has been deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) on June 30, 1998 under the accession number I-2049.

9. A method of isolating a polynucleotide of interest that is present in a genome of a first mycobacterium strain or that is expressed by the first mycobacterium strain and that is absent or altered in a genome of a second mycobacterium strain or that is not expressed by the second mycobacterium strain, said method comprising :

a) providing at least one polynucleotide contained in a clone of a bacterial artificial chromosome (BAC) DNA library of the first mycobacterium strain;

b) providing at least one genomic or cDNA polynucleotide from a second mycobacterium strain that is different from the first mycobacterium strain or at least one polynucleotide contained in a clone of a BAC DNA library prepared from the genome of the second mycobacterium strain;

c) contacting under hybridizing conditions the polynucleotide of step a) with the polynucleotide of step b); and

d) isolating the polynucleotide of step a) that has not formed a hybrid complex with the polynucleotide of step b).

10. The method of claim 9, wherein the polynucleotide contained in a clone of a BAC DNA library of the first or second mycobacterium strain is prepared by the following procedure :

1) digesting at least one recombinant BAC clone by an appropriate restriction endonuclease to yield a polynucleotide insert of interest; and

2) isolating the polynucleotide insert of interest.

11. A purified polynucleotide of interest that has been isolated according to the method of claim 9.

12. The purified polynucleotide of claim 11 which contains at least one Open Reading Frame (ORF).

13. The purified polynucleotide of claim 12, which is SEQ ID N0:1.

14. The purified polynucleotide of claim 12, wherein said polynucleotide is selected from the group consisting of :

a) a polynucleotide comprising at least 8 consecutive nucleotides of SEQ ID N0:1;

b) a polynucleotide having a sequence fully complementary to SEQ ID N0:1; and

c) a polynucleotide that hybridizes under stringent hybridization conditions with the polynucleotide defined in a) or with the polynucleotide defined in b).

15. The purified polynucleotide of claim 14, which is SEQ ID N0:2.

16. The purified polynucleotide of claim 14, which is SEQ ID N0:3.

17. The purified polynucleotide of claim 12, wherein the ORF encodes all or part of a polypeptide involved in the pathogenicity of a mycobacterium strain.

18. The purified polynucleotide of claim 12, wherein the ORF encodes all or part of a Polymorphism Glycine Rich Sequence (PGRS).

19. The purified polynucleotide of claim 18, which is SEQ ID N0:4.

20. The purified polynucleotide of claim 18, which is selected from the group consisting of:

a) a polynucleotide comprising at least 8 consecutive nucleotides the of SEQ ID N0:5 ;

b) a polynucleotide having a sequence that is fully complementary to SEQ ID N0:5 ;

c) a polynucleotide that hybridizes under stringent hybridization conditions with the polynucleotide defined in a) or with the polynucleotide defined in b).

21. A pair of the purified polynucleotides as claimed in claim 11.

22. A *Mycobacterium tuberculosis* strain Rv37 genomic DNA library that has been deposited in the Collection Nationale de Cultures de Microorganismes under accession number I-1945, wherein said genomic DNA library comprises recombinant bacterial artificial chromosome vectors.

23. A recombinant bacterial artificial chromosome (BAC) vector, which belongs to the genomic DNA library of claim 22.

24. The recombinant BAC vector of claim 23, which is selected from the group consisting of :

Rv101; Rv102; Rv103; Rv104; Rv105; Rv106; Rv107; Rv108; Rv109; Rv10; Rv110; Rv111; Rv112; Rv113; Rv114; Rv115; Rv116; Rv117; Rv118; Rv119; Rv11; Rv120; Rv121; Rv122; Rv123; Rv124; Rv126; Rv127; Rv128; Rv129; Rv130; Rv132; Rv134; Rv135; Rv136; Rv137; Rv138; Rv139; Rv13; Rv140; Rv141; Rv142; Rv143; Rv144; Rv145; Rv146; Rv147; Rv148; Rv149; Rv14; Rv150; Rv151; Rv152; Rv153; Rv154; Rv155; Rv156; Rv157; Rv159; Rv15; Rv160; Rv161; Rv162; Rv163; Rv164; Rv165; Rv166; Rv167; Rv169; Rv16; Rv170; Rv171; Rv172; Rv173; Rv174; Rv175; Rv176; Rv177; Rv178; Rv179; Rv17; Rv180; Rv181; Rv182; Rv183; Rv184; Rv185; Rv186; Rv187; Rv188; Rv18; Rv190; Rv191; Rv192; Rv193; Rv194; Rv195; Rv196; Rv19; Rv1; Rv201; Rv204; Rv205; Rv207; Rv209; Rv20; Rv214; Rv215; Rv217; Rv218; Rv219; Rv21; Rv220; Rv221; Rv222; Rv223; Rv224; Rv225; Rv226; Rv227; Rv228; Rv229; Rv22; Rv230; Rv231; Rv232; Rv233; Rv234; Rv235; Rv237; Rv240; Rv241; Rv243; Rv244; Rv245; Rv246; Rv247; Rv249; Rv24; Rv251; Rv252; Rv253; Rv254; Rv255; Rv257; Rv258; Rv259; Rv25; Rv260; Rv261; Rv262; Rv263; Rv264; Rv265; Rv266; Rv267; Rv268; Rv269; Rv26; Rv270; Rv271; Rv272; Rv273; Rv274; Rv275; Rv276; Rv277; Rv278; Rv279; Rv27; Rv280; Rv281; Rv282; Rv283; Rv284; Rv285; Rv286; Rv287; Rv288; Rv289; Rv28; Rv290; Rv291; Rv292; Rv293; Rv294; Rv295; Rv296; Rv29; Rv2; Rv301; Rv302; Rv303; Rv304; Rv306; Rv307; Rv308; Rv309; Rv30; Rv310; Rv311; Rv312; Rv313; Rv314; Rv315; Rv316; Rv317; Rv318; Rv319; Rv31; Rv32; Rv322; Rv327; Rv328; Rv329; Rv32; Rv330; Rv331; Rv333; Rv334; Rv335; Rv336; Rv337; Rv338; Rv339; Rv33; Rv340; Rv341; Rv343; Rv344; Rv346; Rv347; Rv348; Rv349; Rv34; Rv350; Rv351; Rv352; Rv353; Rv354; Rv355; Rv356; Rv357; Rv358; Rv359; Rv35; Rv360; Rv361; Rv363; Rv364; Rv365; Rv366; Rv367; Rv368; Rv369; Rv36; Rv370; Rv371; Rv373; Rv374; Rv375; Rv376; Rv377; Rv378; Rv379; Rv37; Rv381; Rv382; Rv383; Rv384; Rv385; Rv386; Rv387; Rv388; Rv389; Rv38; Rv390; Rv391; Rv392; Rv393; Rv396; Rv39; Rv3; Rv40; Rv412; Rv413; Rv414; Rv415; Rv416; Rv417; Rv418; Rv419;

Rv41; Rv42; Rv43; Rv44; Rv45; Rv46; Rv47; Rv48; Rv49; Rv4; Rv50; Rv51; Rv52; Rv53; Rv54; Rv55; Rv56; Rv57; Rv58; Rv59; Rv5; Rv60; Rv61; Rv62; Rv63; Rv64; Rv65; Rv66; Rv67; Rv68; Rv69; Rv6; Rv70; Rv71; Rv72; Rv73; Rv74; Rv75; Rv76; Rv77; Rv78; Rv79; Rv7; Rv80; Rv81; Rv82; Rv83; Rv84; Rv85; Rv86; Rv87; Rv88; Rv89; Rv8; Rv90; Rv91; Rv92; Rv94; Rv95; Rv96 and Rv9.

25. The recombinant BAC vector of claim 23, which is selected from the group consisting of:

Rv234; Rv351; Rv166; Rv35; Rv415; Rv404; Rv209; Rv272; Rv30; Rv228; Rv233; Rb38; Rv280; Rv177; Rv48; Rv374; Rv151; Rv238; Rv156; Rv92; Rv3; Rv403; Rv322; Rv243; Rv330; Rv285; Rv233; Rv219; Rv416; Rv67; Rv222; Rv149; Rv279; Rv87; Rv273; Rv266; Rv25; Rv136; Rv414; Rv13; Rv289; Rv60; Rv104; Rv5; Rv165; Rv215; Rv329; Rv240; Rv19; Rv74; Rv411; Rv167; Rv56; Rv80; Rv164; Rv59; Rv313; Rv265; Rv308; Rv220; Rv258; Rv339; Rv121; Rv419; Rv418; Rv45; Rv217; Rv134; Rv17; Rv103; Rv21; Rv22; Rv2; Rv270; Rv267; Rv174; Rv257; Rv44; Rv71; Rv7; Rv27; Rv191; Rv230; Rv128; Rv407; Rv106; Rv39; Rv255; Rv74; Rv355; Rv268; Rv58; Rv173; Rv264; Rv417; Rv401; Rv144; Rv302; Rv81; Rv163; Rv281; Rv221; Rv420; Rv175; Rv86; Rv412; Rv73; Rv269; Rv214; Rv287; Rv42 and Rv143.

26. A *Mycobacterium bovis* BCG strain Pasteur genomic DNA library, wherein said genomic DNA library comprises recombinant bacterial artificial chromosome vectors.

27. A *Mycobacterium bovis* BCG strain Pasteur genomic DNA library according to claim 26, wherein said DNA library contains approximatively 1600 clones and wherein the genomic DNA is cloned into a recombinant pBeloBAC11 vector with an average insert size of approximately 80 kb.

28. A *Mycobacterium bovis* BCG strain Pasteur genomic DNA library according to claim 26, that has been deposited in the Collection Nationale de

Cultures de Microorganismes (CNCM) on June 30, 1998 under the accession number I-2049.

29. A recombinant bacterial artificial chromosome (BAC) vector, which belongs to the genomic DNA library of claims 26 to 28.

30. A recombinant BAC vector according to claim 29, which is selected from the group consisting of:
X0001; X0002; X0003; X0004; X0006; X0007; X0008; X0009; X0010; X0012; X0013; X0014; X0015; X0016; X0017; X0018; X0019; X0020; X0021 and X0175.

31. A method for detecting a mycobacterial nucleic acid in a biological sample comprising the steps of:
a) contacting the recombinant BAC vector according to claim 23 or 29, or a purified polynucleotide according to claim 11 with the mycobacterial nucleic acid in the biological sample ; and
b) detecting a hybrid nucleic acid molecule formed between said recombinant BAC vector or said purified polynucleotide and the mycobacterial nucleic acid in the biological sample.

32. The method of claim 31, further comprising before step a), making the mycobacterial nucleic acid in the biological sample available to a hybridization reaction.

33. A method for detecting mycobacterial nucleic acid in a biological sample comprising the steps of:
a) contacting a first polynucleotide according to claim 11 that has been immobilized onto a substrate with the mycobacterial nucleic acid in the biological sample ; and
b) contacting a hybrid nucleic acid molecule formed between said first polynucleotide and the mycobacterial nucleic acid in the biological sample with a second, labeled polynucleotide according to claim 11, wherein said

second polynucleotide and said first polynucleotide have non-overlapping sequences.

34. The method of claim 33, further comprising before step a), making the mycobacterial nucleic acid in the biological sample available to a hybridization reaction.

35. The method of claim 33 or 34, further comprising before step b), removing the mycobacterial nucleic acid that is not hybridized with the immobilized first polynucleotide.

36. A method for detecting mycobacterial nucleic acid in a biological sample comprising the steps of:

a) contacting the mycobacterial nucleic acid in the biological sample with a pair of purified polynucleotides according to claim 21 ;

b) amplifying said mycobacterial nucleic acid ; and

c) detecting the amplified mycobacterial nucleic acid.

37. The method of claim 36, further comprising before step a), making the mycobacterial nucleic acid in the biological sample available to a hybridization reaction.

38. A kit for detecting a mycobacterium in a biological sample comprising:

a) a recombinant BAC vector according to claim 23 or 29, or a purified polynucleotide according to claim 11 ; and

b) reagents necessary to perform a nucleic acid hybridization reaction.

39. A kit for detecting a mycobacterium in a biological sample comprising:

a) a recombinant BAC vector according to claim 23 or 29, or a first polynucleotide according to claim 11 that is immobilized onto a substrate ;

b) reagents necessary to perform a nucleic acid hybridization reaction ; and

c) a second polynucleotide according to claim 11, wherein said second polynucleotide is radioactively or non-radioactively labeled, and wherein said second polynucleotide and said first polynucleotide have non-overlapping sequences.

40. A kit for detecting a mycobacterium in a biological sample comprising:

a) a pair of purified polynucleotides according to claim 20 ; and

b) reagents necessary to perform a nucleic acid amplification reaction.

41. A method for detecting the presence of a genomic DNA, a cDNA or a mRNA of a mycobacterium in a biological sample, comprising the steps of:

a) contacting the biological sample with a plurality of BAC vectors according to claim 23 or 29, or purified polynucleotides according to claim 11 that are immobilized on a substrate ; and

b) detecting the hybrid complexes formed.

42. A kit for detecting a genomic DNA, a cDNA or a mRNA of a mycobacterium in a biological sample, comprising:

a) a substrate on which a plurality of BAC vectors according to claim 23 or 29, or purified polynucleotides according to claim 11 have been immobilized.

43. A method for detecting a polynucleotide of mycobacterial origin in a biological sample, said method comprising:

a) aligning at least one polynucleotide contained in a recombinant BAC vector according to claim 23 or 29 on the surface of a substrate ;

b) contacting the polynucleotide in the biological sample with the substrate on which the polynucleotide of step a) has been aligned ; and

c) detecting a hybrid nucleic acid molecule formed between the polynucleotide in the biological sample and the aligned polynucleotide of step a).

44. A kit for detecting a polynucleotide of mycobacterial origin in a biological sample, comprising:

a) a substrate on which at least one polynucleotide contained in a recombinant BAC vector according to claim 23 or 29 has been aligned.

45. The method of claim 10, wherein the procedure by which the polynucleotide contained in a clone of a BAC DNA library is prepared, further comprises amplifying the polynucleotide insert.

46. The method of claim 10, wherein the procedure by which the polynucleotide contained in a clone of a BAC DNA library is prepared, further comprises digesting the polynucleotide insert with at least one restriction endonuclease.

47. The method of claim 45, further comprising digesting the amplified polynucleotide insert with at least one restriction endonuclease.

48. The Polynucleotide of claim 17, wherein the mycobacterium strain is *Mycobacterium tuberculosis*.

49. The method of claim 36, wherein the amplified mycobacterial DNA is detected by gel electrophoresis or with a labeled polynucleotide according to claim 11.

50. The kit of claim 40, further comprising a polynucleotide according to claim 11.

51. The kit of claim 42, further comprising reagents necessary to perform a hybridization reaction.

52. A method for physically mapping a polynucleotide of mycobacterial origin in a biological sample, said method comprising:

a) aligning at least one polynucleotide contained in a recombinant BAC vector according to claim 23 or 29 on the surface of a substrate;

b) contacting the polynucleotide in the biological sample with the substrate on which the polynucleotide of step a) has been aligned under hybridizing conditions; and

c) detecting the location of the hybridized polynucleotide from the biological sample.

53. The kit of claim 44, further comprising reagents necessary for labeling DNA and reagents necessary for performing a hybridization reaction.

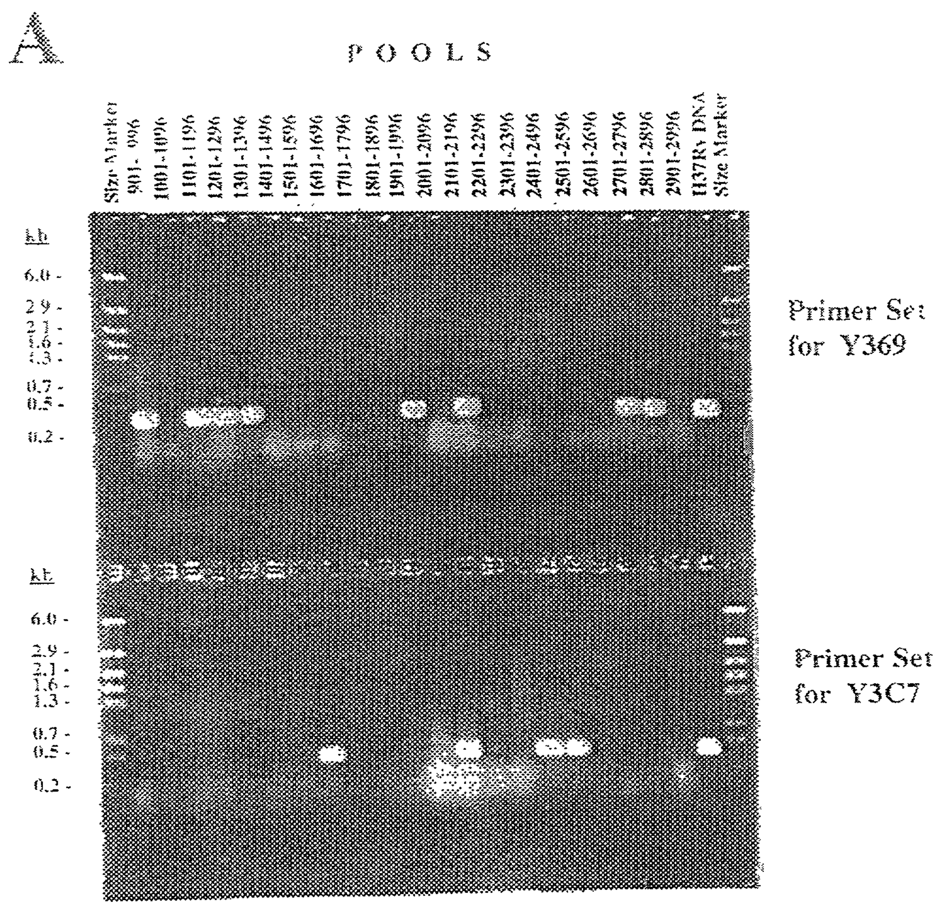
A
POOLS
Size Marker
901-996
1001-1096
1101-1196
1201-1296
1301-1396
1401-1496
1501-1596
1601-1696
1701-1796
1801-1896
1901-1996
2001-2096
2101-2196
2201-2296
2301-2396
2401-2496
2501-2596
2601-2696
2701-2796
2801-2896
2901-2996
H37Rv DNA
Size Marker
kb
6.0 -
2.9 -
2.1 -
1.6 -
1.3 -
0.7 -
0.5 -
0.2 -
Primer Set for Y369
kb
6.0 -
2.9 -
2.1 -
1.6 -
1.3 -
0.7 -
0.5 -
0.2 -
Primer Set for Y3C7

FIGURE 1A

B

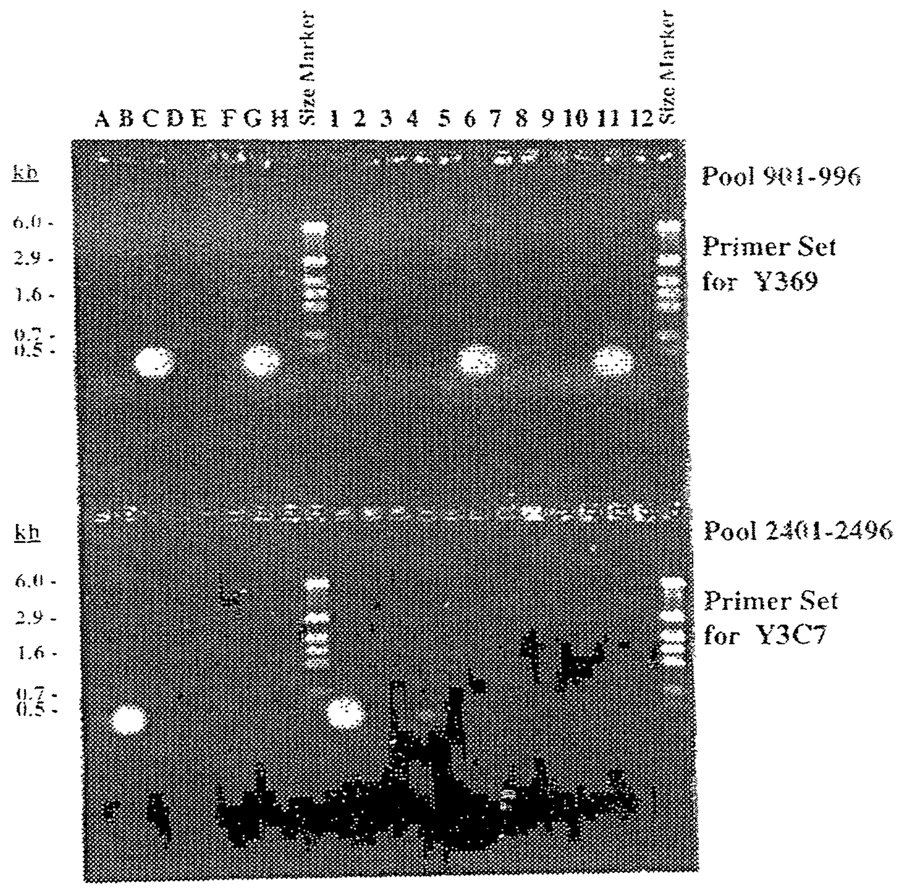
A B C D E F G H Size Marker 1 2 3 4 5 6 7 8 9 10 11 12 Size Marker
kb
6.0 -
2.9 -
1.6 -
0.7 -
0.5 -
Pool 901-996
Primer Set
for Y369
kb
6.0 -
2.9 -
1.6 -
0.7 -
0.5 -
Pool 2401-2496
Primer Set
for Y3C7

FIGURE 1B

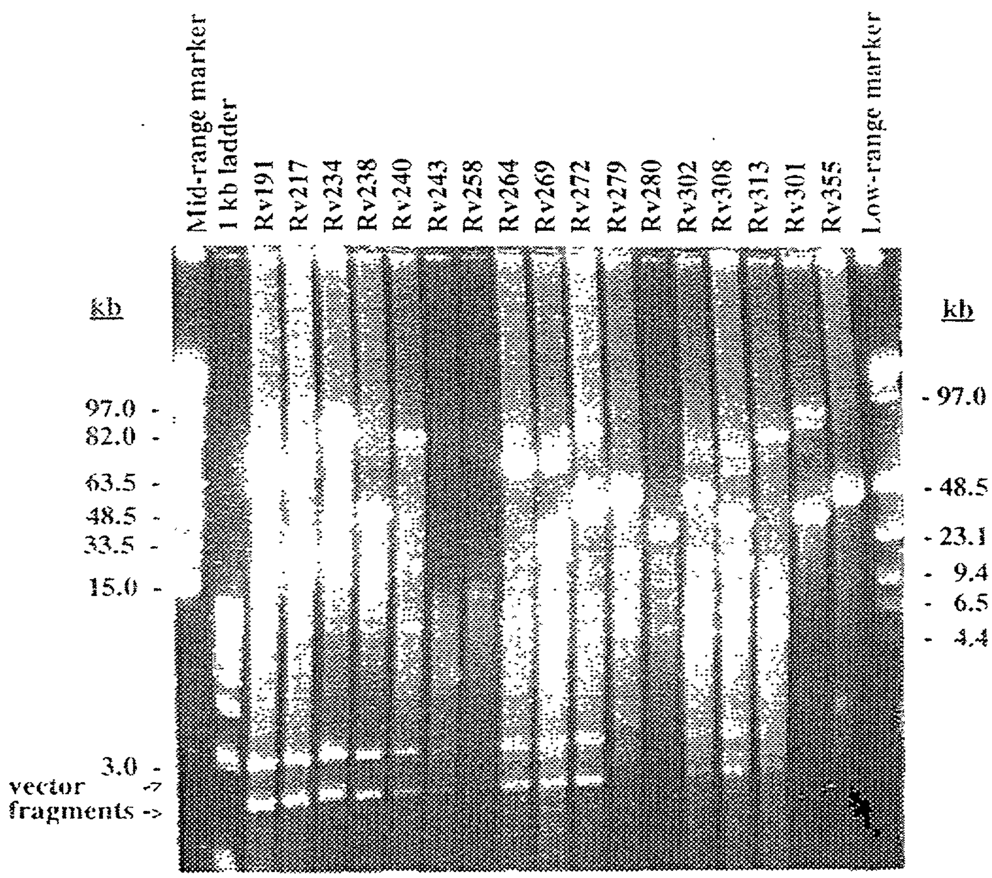
Mid-range marker
1 kb ladder
Rv191
Rv217
Rv234
Rv238
Rv240
Rv243
Rv258
Rv264
Rv269
Rv272
Rv279
Rv280
Rv302
Rv308
Rv313
Rv301
Rv355
Low-range marker
kb
97.0 -
82.0 -
63.5 -
48.5 -
33.5 -
15.0 -
3.0 -
vector ->
fragments ->
kb
- 97.0
- 48.5
- 23.1
- 9.4
- 6.5
- 4.4

FIGURE 2

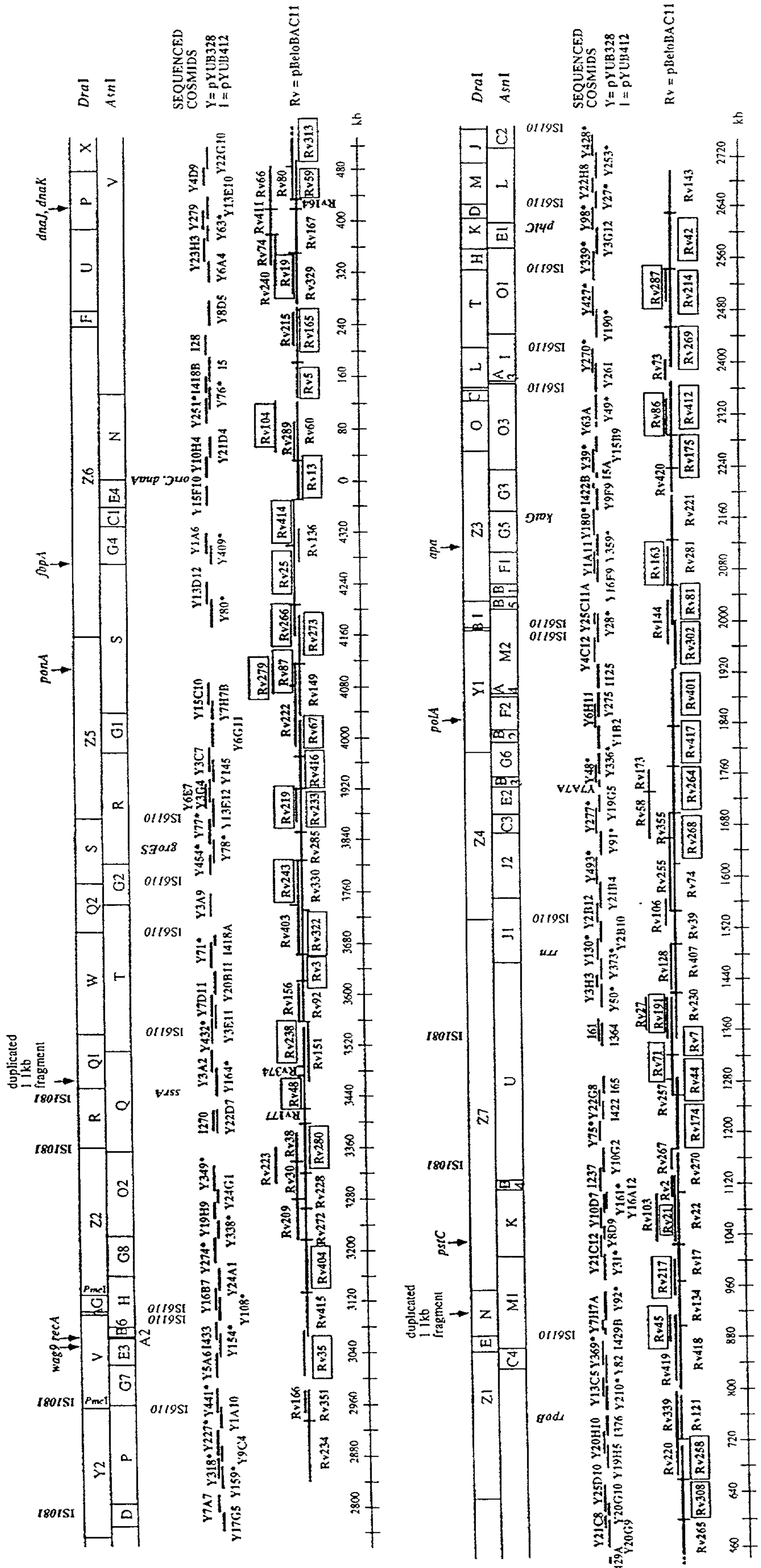

FIGURE 3

A

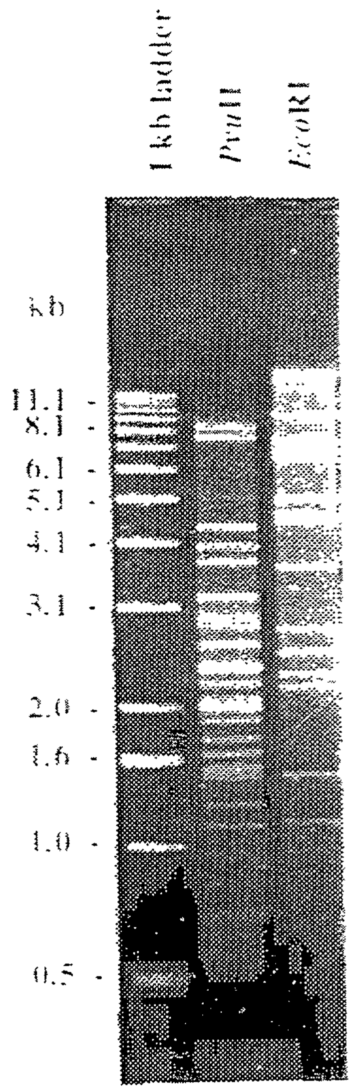

FIGURE 4A

B

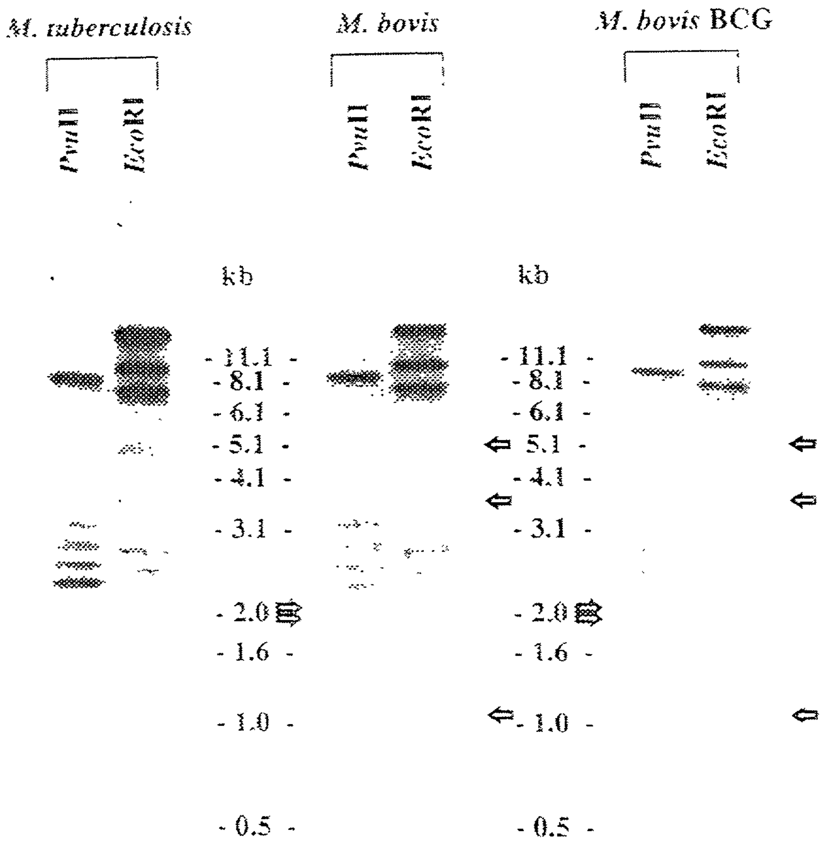
M. tuberculosis
M. bovis
M. bovis BCG
PvuII
EcoRI
PvuII
EcoRI
PvuII
EcoRI
kb
kb
- 11.1 -
- 8.1 -
- 6.1 -
- 5.1 -
- 4.1 -
- 3.1 -
- 2.0
- 1.6 -
- 1.0 -
- 0.5 -
- 11.1 -
- 8.1 -
- 6.1 -
5.1 -
- 4.1 -
- 3.1 -
- 2.0
- 1.6 -
- 1.0 -
- 0.5 -

FIGURE 4B

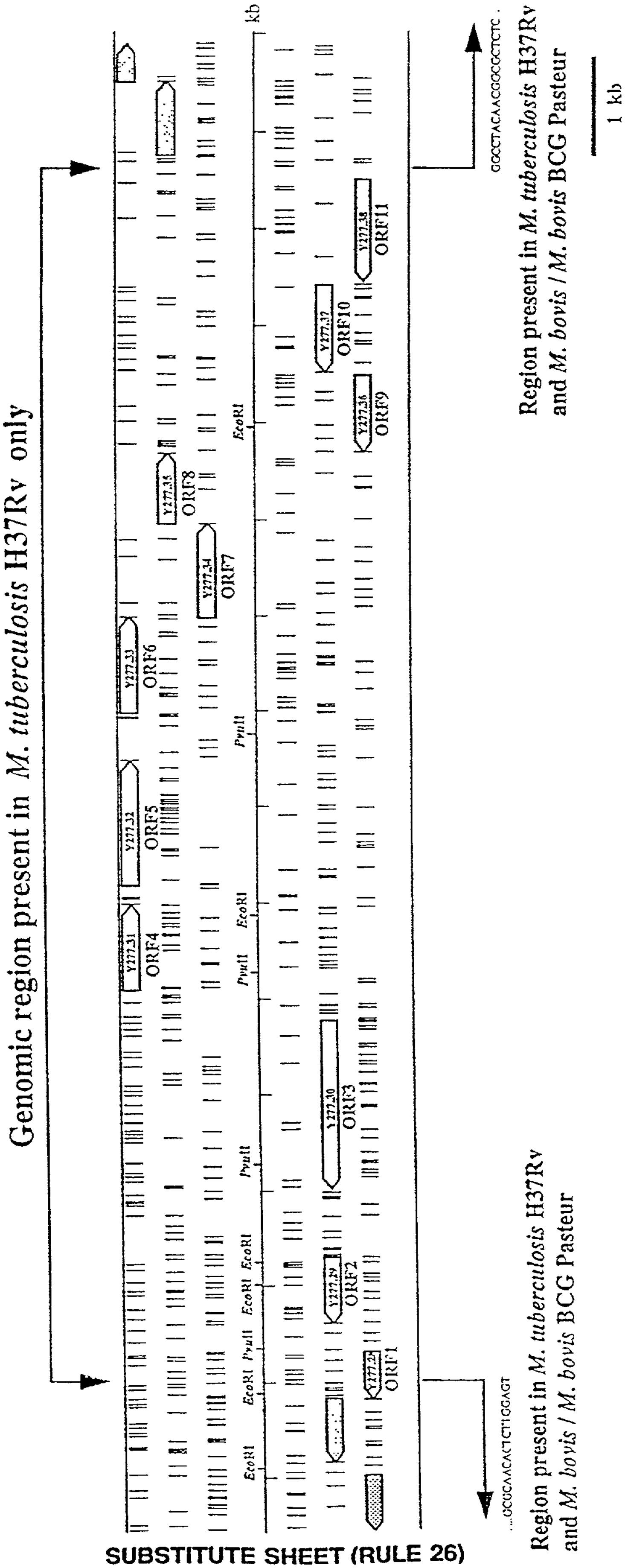
Genomic region present in M. tuberculosis H37Rv only
Region present in M. tuberculosis H37Rv and M. bovis / M. bovis BCG Pasteur
Region present in M. tuberculosis H37Rv and M. bovis / M. bovis BCG Pasteur
1 kb
kb
ORF1
ORF2
ORF3
ORF4
ORF5
ORF6
ORF7
ORF8
ORF9
ORF10
ORF11
EcoRI
PvuII

FIGURE 5

```
H37Rv  ...PTQTLTGRPLIGNGTPGAVGSGATGAPGGWLLGDGGAGGSGAAGSGAPGGAGGAAGLWGT 837273
BCG    ...PTQTLTGRPLIGNGTPGAVGSGATGAPGGWLLGDGGAGGSGAAGSGAPGGAGGAAGLWGT

H37Rv  GGAGGAGGSSAGGGGAGGAGGAGGWLLGDGGAGGIGGASTVLGGTGGGGVGGLWGAGGA 837453
BCG    -----------------------------GGAGGIGGASTVLGGTGGGGVGGLWGAGGA

H37Rv  GGAGGTGLVGGDGGAGGAGGTGGLLAGLIGAGGGHGGTGGLSTNGDGGVGGAGGNAGMLA 837633
BCG    GGAGGTGLVGGDGGAGGAGGTGGLLAGLIGAGGGHGGTGGLSTNGDGGVGGAGGNAGMLA

H37Rv  GPGGAGGAGGDGEMLDTGGDGGAGGSAGLLFGSGGAGGAGGFGFLGGDGGAGGNAGLLLS 837813
BCG    GPGGAGGAGGDGEMLDTGGDGGAGGSAGLLFGSGGAGGAGGFGFLGGDGGAGGNAGLLLS

H37Rv  SGGAGGFGGFGTAGGVGGAGGNAGWLGF 837897 --------------------------------
BCG    SGGAGGFGGFGTAGGVGGAGGNAGWLGFGAGGIGGIGGNANGGAGGNGGTGGQLWGSGGA

H37Rv  -------------GGAGGVGGSAGLIGTGGNGGNGGTGANAGSPGTGGAGGLLLGQNGLNGLP 838047
BCG    GVEGGAALSVGDTGGAGGVGGSAGLIGTGGNGGNGGTGANAGSPGTGGAGGLLLGQNGLNGLP
```

FIGURE 6

pBeloBAC11

GCGGCCGCAA GGGGTTCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG
*Not*I restriction site

CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG

GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC

ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC

TCTTCGCTAT TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT
primer T7-BAC1

AAGTTGGGTA ACGCCAGGGT TTTCCCAGTC ACGACGTTGT AAAACGACGG

CCAGTGAATT GTAATACGAC TCACTATAGG GCGAATTCGA GCTCGGTACC
T7-promoter sequence

CGGGGATCCT CTAGAGTCGA CCTGCAGGCA TGCAAGCTTG AGTATTCTAT
primer T7-Belo2 *Hind*III cloning site SP6-promoter

AGTGTCACCT AAATAGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG
sequence (complementary strand) primer SP6-Mid (complementary strand)

TGAAATTGTT ATCCGCTCAC AATTCCACAC AACATACGAG CCGGAAGCAT

AAAGTGTAAA GCCTGGGGTG CCTAATGAGT GAGCTAACTC ACATTAATTG
primer SP6-BAC1 (complementary strand)

CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG GAAACCTGTC GTGCCAGCTG

CATTAATGAA TCGGCCAACG CGAACCCCTT GCGGCCGCCC GGGCCGTCGA
*Not*I restriction site

FIGURE 7

#3

Attorney Docket No. ____________________

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: A method for isolating a polynucleotide of interest from the genome of a mycobacterium using a BAC-based DNA library. Application to the detection of mycobacteria

the specification of which ☐ is attached and/or ☒ was filed on April 16, 1999 as United States Application Serial No. ____________ or PCT International Application No. PCT/IB99/00740 and was amended on ____________ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
US	09/060 756	16/04/1998	☐ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application.

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)

I hereby appoint the following attorney and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P., Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilley, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewris, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220; Walter Y. Boyd, Jr., Reg. No. 31,738; Steven M. Anzalone, Reg. No. 32,095; Jean B. Fordis, Reg. No. 32,984; Barbara C. McCurdy, Reg. No. 32,120; James K. Hammond, Reg. No. 31,964; Richard V. Burgujian, Reg. No. 31,744; J. Michael Jakes, Reg. No. 32,824; Dirk D. Thomas, Reg. No. 32,600; Thomas W. Banks, Reg. No. 32,719; Christopher P. Isaac, Reg. No. 32,616; Bryan C. Diner, Reg. No. 32,409; M. Paul Barker, Reg. No. 32,013; Andrew Chanho Sonu, Reg. No. 33,457; David S. Forman, Reg. No. 33,694; Vincent P. Kovalick, Reg. No. 32,867; James W. Edmondson, Reg. No. 33,871; Michael R. McGurk, Reg. No. 32,045; Joann M. Neth, Reg. No. 36,363; Gerson S. Panitch, Reg. No. 33,751; Cheri M. Taylor, Reg. No. 33,216; Charles E. Van Horn, Reg. No. 40,266; and Linda A. Wadler, Reg. No. 33,218; and ____________________. Please address all correspondence to FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P. 1300 I Street, N.W., Washington, D.C. 20005, Telephone No. (202) 408-4000.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Attorney Docket No. ____________

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09/673476
528 Rec'd PCT/PTO 16 OCT 2000

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: INSTITUT PASTEUR
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(F) POSTAL CODE (ZIP): 75724

(ii) TITLE OF INVENTION: A METHOD FOR ISOLATING A POLYNUCLEOTIDE OF INTEREST FROM THE GENOME OF A MYCOBACTERIUM USING A BAC-BASED DNA LIBRARY. APPLICATION TO THE DETECTION OF MYCOBACTERIA.

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12732 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GCTCGGACTG CCAATACGCG AAGCCTGGTT CACACCCGGA CAGGTATGCC ACCACGTCGT 10440

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CACTTTTCAC CGTGCGCTGC AACCCGTCGA GGTCGTTGAA CGAGATCGTT ATCACCGAGA 10560

CGGTCGGAGC AGACGTCACC GAGTTCCCCT AGGTTGCTGG CGGCGATTGT GGATCACCGG 10620

GTCTTGATAC CGATGAAGGT GCCTCGAAGA TTCGCCGCAT AGGAACCTCC GAGCAACGAC 10680

TCGGCGATGC TTGGTTCCAA GTTGTCGTAC TCCTCCATCA CCAGGTCGAC GCCGACGTCT 10740

TTGATGGCCT GAAGTAGGTG CTCGCGTTGA ATCCAGAATG ACCGGCGATT GTCCCAGGAC 10800

GCCCATTTTG CGGTGTCGCG CTGGCCAAAC GAGCGGTCGT CGGAAAACTC GGTAAACCAC 10860

CTACCGGGAA GTCCCTCATG TTCGGTGGGC GCCGAGAGCA TGAACTTCAC CGGCGCCGGC 10920

CGCCGCAGCA ACCGATCGGT CAATTGTCGT GCCGTCGTGG GCAACCGGAG CCATTTATCG 10980

CTCCGGTTGA TGATCGAGAA GTGCGTCTGG AGAATCAGCA GCTTGTTCGT TACCGACGAG 11040

AGGGTTTCCA GGTATTGCTT CGGATTCTCC AGGTGGTAGA AGAGGCCGCA GCAGAAGACG 11100

GTATCGAAGA GCCCGTGGTT GGCGATGTTG AGGGCGTTGT CGTGGACGAA CCGGAGATTC 11160

GGCAGGTTGG TCTTCGATTT GATGTAGTTG CAGGCCGCCA TGTTCAGCTC GCGAACCTCG 11220

ATCCCGAGGA CCTGAAATCC CATGCGCGCG AACCCGACCG CGTACCCGCC TTCCAAGCAG 11280

CCGACATCGG CCAGGCGTAG GTGGCTCTTG TCCCCGGGAA AGACGGTTTC CAGAATCCCG 11340

CGCGCCGAGA TGAACCAGGA CGATTCGTCT AACGTGCGCG AGGACTCCGG TATCGTCAAG 11400

GTTCCGTCGT CGAGGCGAAC GTTGTGGGCG GTGAATTGTA CCGCGCCGGC CGAATGTTCC 11460

TGTGCCATCA CTTGGTTAGC CCCTTCGGCT GGTCCTGGGT TTGTCGACAT GGTCAGGCTC 11520

GACAGCCGCG TCGGAGCCGG GAGGGCCACA CATCCACGAG CCCCCTGCGG CTCGGCGTCG 11580

CGGCGGCGAG CTTGCGCCAC TGGGTCTTGA GCCGCCGCGC GGGTGTCGCC CCGCGGTGCT 11640

GCAGCGCCAG CATGGCGATC CGGGGATGGC GCGCGATGGT TTCCTGCAGC GCGGCGCGCC 11700

CCTCCGGGCC TGGAACGTTG GCGATCTGGC GAAGGATCCA GTCGGCCATG ACGGCGATGA 11760

GCTCCTCGCG CGCGGGGTCT CCCGGGAACA GGTCGAGCAT CGCGTCAAAC GTCGCCGCAT 11820

GCCCCGGACC CTGCGTCAAC CAGAACTTTG GCGGGTCCAC CACCTGGTTG TGCCACATGC 11880

CTTGGGCGTG GCGGCGATAC ACGGCCATGG TGTCGGGCAA CATGGCGATG TCGCCATGCA 11940

CCGCGTGCCG GACGTGCAGA TACCAGTCCA GGGGCATGAC GTCGGCAGGA ATGTCGTCGT 12000

AGCGCTCGAG GCGACGGTAC ACGGCCGAGT TGGTCTGGAT GAAGTTCATC AAGATCAACG 12060

CATCCAGGCT CAAGTTGCCC CGCACCCGAA CCGGGGGGAA CTTCGAGTCC TTGGCATGGC 12120

CGTCCTCCCA TATCACTCGG ACGGGATGGA AGCACACCGT CGTCTTGGGG TGCCGGTCGA 12180

GGAATGCGAC CTGTTTGCTT AGCTTCAGCG GATCGATCCA GTAGTCGTCC GCCTCGCACA 12240

ACGCGACGTA CTCGCCGCGA GCGGCCGACA GGGCGCCGGT CAGGTTCCCA TTGAGGCCGA 12300

GGTTTTCGGT CCTGAAGATC GGCCGGAACA CGTGCGGGTA CCGCTCGGCG TACTCACGGA 12360

TGATCGCCGG GGTGGCATCG GTCGACGCGT CGTCGGCGAC GATGATCTCC ACCGGGAAGT 12420

CGGTTTGCTG GTCGAGAAAG CTGTCGAAGG CCTGACGGGC GTAGCCCGCC TGGTTGTGAG 12480

TGGTCGAGAC GATGCTCACC TTGGGGCAAA GCTGGGGACT CACCGTCGGC CCTTTTCCTG 12540

CGCGGCCGCA AGGGTATTGC GATGGCGAAC GTGAATCGCC TGTGCCCGCC GGCCGTCGGC 12600

CGTCGTGGCC TGGTGGTCGG CGGACGTACG GCACACGCTG GCGAAGTATA GCGAGGGTGC 12660

ACTGACGTTG GGCTCGAACC GCGTGGCGCG CGGTGTGGGC GCACCGTCTC GAGTCGGTGC 12720

TGGTTGGCTC GC 12732

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 289 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATACTCAAGC TTGCCGCAAT CGAAACCAAC CTGTTTGTGC CGCAAGAAAT TACGCCGTGG 60

CCCGGCGCCG ATCAAGAAAC GCCCCGGCGC GCGGCGGTGT CGTCGTATGG CATGACGGGC 120

ACCAATGTGC ACGCCATTGT CGAGCAGGCA CCGGTGCCAG CCCCCGAATC CGGTGCACCA 180

GGCGACACCC CGGCCACACC CGGTATCGAC GGCGCGCTGC TGTTCGCGCT GTCGGCCAGC 240

TCGCAGGACG CGCTGCGGCA AACCGCCGCG CGGCTGGCCG ATTGGGTCT 289

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 278 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTGGCGGGTT GGCCACACAC CCGCCGGTGA CGGCGACGAT GCTGGGCTGG TTGCGGCCCT 60

GCGCCACCGC GGCTTGCATG CTGGTTGGCT GTCTTGGGAC GATCCCGAAA TAGTCCACGC 120

GGATCTGGTG ATTTTGCGGG CTACCCGCGA TTACCCCGCG CGGCTCGACG AGTTTTTGGC 180

CTGGACTACC CGCGTGGCCA ATCTGCTGAA CTCGCGGCCG GTGGTGGCCT GGAATGTCCA 240

CGCCGTTCAC CTACGTGACC TTGATGGGAT CCGGGGGT 278

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1280 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCGACCCAGA CACTGACCGG GCGACCGCTG ATCGGCAACG GCACCCCCGG GGCGGTCGGC 60

AGCGGGGCCA CCGGGGCCCC CGGTGGGTGG CTGCTCGGCG ACGGCGGGGC CGGCGGGTCC 120

GGCGCGGCGG GCTCGGGCGC GCCCGGCGGG GCGGGCGGGG CTGCCGGGCT GTGGGGTACC 180

GGCGGGGCCG GCGGGATCGG CGGAGCCAGC ACCGTACTCG GCGGCACCGG CGGGGGAGGC 240

GGGGTCGGTG GGCTGTGGGG CGCCGGTGGG GCCGGCGGGG CCGGTGGAAC CGGCCTTGTT 300

GGTGGCGACG GCGGGGCCGG TGGGGCCGGC GGGACCGGCG GACTGCTGGC CGGGCTGATC 360

GGTGCCGGCG GAGGTCACGG CGGGACCGGC GGGCTCAGCA CTAATGGCGA CGGCGGGGTT 420

GGCGGGGCCG GCGGGAATGC CGGAATGCTC GCCGGGCCGG GCGGCGCCGG CGGAGCCGGC 480

GGTGACGGCG AAAACCTGGA CACCGGTGGG GACGGCGGGG CCGGCGGTAG CGCAGGGCTG 540

CTGTTCGGCA GCGGCGGCGC CGGCGGCGCC GGCGGATTTG GTTTCCTCGG TGGGGACGGC 600

GGGGCCGGTG GCAACGCCGG GCTGCTGTTG TCCAGCGGCG GGGCCGGCGG GTTCGGCGGG 660

TTCGGCACCG CCGGTGGGGT CGGTGGGGCC GGCGGCAATG CCGGCTGGCT GGGCTTCGGC 720

GGGGCCGGGG GCATCGGCGG AATCGGCGGT AACGCTAACG GGGGCGCCGG TGGGAACGGC 780

GGCACCGGCG GTCAGTTATG GGGTAGCGGC GGCGCCGGCG TCGAAGGCGG CGCAGCCTTA 840

AGCGTCGGCG ACACCGGCGG GGCCGGTGGC GTCGGCGGCA GCGCCGGGCT GATCGGCACC 900

GGCGGCAACG GCGGCAACGG CGGCACCGGC GCCAACGCCG GCAGCCCCGG AACCGGCGGC 960

GCCGGCGGGT TGCTGCTGGG CCAAAACGGG CTCAACGGGT TGCCGTAGCC GGGCGGCACG 1020

GCATGGCTTC CGGGCGTCAA CCACTCGCCG GTGATGCAGA TCGGCTGCGG AGCGGGCCGC 1080

CAAAATGGGG GCCGCCGCGC CAGGTATCTC GGCGAAGATC CCCGGCGCTC GAGCGCTTTG 1140

TCAGAGGCCC GTCGCGGGTC GTCGTGACGA CGGCTATCCG GGCGGTGCGG GTTTCGCGGC 1200

GCGCCCTGTG CCCGGCACCG CCGCCCGTTT GTCGGCAACG CCGCCGCGAC CCGTGAGCCG 1260

TCCAGCAGCT GGCGCCTGCG 1280

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GGGCATCGGC GGAATCGGCG GTAACGCTAA CGGGGGCGCC GGTGGGAACG GCGGCACCGG 60

CGGTCAGTTA TGGGGTAGCG GCGGCGCCGG CGTCGAAGGC GGCGCAGCCT TAAGCGTCGG 120

CGACACC 127